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ビールに特徴的な香りを付与する
ホップ由来香気成分

**Hop-Derived Odorants Contributing to
the Aroma Characteristics of Beer**

博士論文
Doctoral Dissertation

2008

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List of Abbreviations

3MH :	3-mercaptohexan-1-ol
3MHA :	3-mercaptohexyl acetate
4MMP :	4-mercapto-4-methylpentan-2-one
AEDA :	aroma extract dilution analysis
AMU :	atomic mass unit
ASBC	American Society of Brewing Chemists
CAS :	Chemical Abstracts Service
EBC :	European Brewery Convention
ee :	enantiomeric excess
FID :	flame-ionization detector
FPD	flame photometric detection
GC :	gas chromatography
GC×GC :	two-dimensional gas chromatography
GC-MS :	gas chromatography coupled with quadrupole-mass spectrometry
GC-O :	GC-olfactometry
HV :	high voltage
ICP-MS :	inductively-coupled plasma mass spectroscopy
MBT:	3-methyl-2-butene-1-thiol
MD :	multidimensional
MS :	mass spectrometer
ND :	not detected
PDMS :	polydimethylsiloxane
<i>p</i> HMB :	<i>p</i> -hydroxymercuribenzoate
PTFE :	polytetrafluoroethylene
RF :	radio frequency
RI :	retention index
SAFE	Solvent-Assisted Flavor Evaporation
SBSE :	stir bar-sorptive extraction
SCD:	sievers chemiluminescence detector
SIM :	select ion monitoring
TDU :	thermal desorption unit
TOFMS:	time-of-flight mass spectrometry

General Introduction

BACKGROUND TO THE CURRENT WORK

Hop plants are grown as an agricultural commodity in various parts of the world, and are almost exclusively used for beer brewing. The common hop (*Humulus lupulus* L.) is a perennial and dioecious climbing plant of the family Cannabaceae (**Figure 0-1a**). The mature female flower cones of the plant, which are known as hops, have been used in the brewing process since mediaeval times to provide a bitter taste and aroma, and to enhance the shelf life of beer. Today, more than 100 hop cultivars are grown throughout the world, and provide various characteristic aromas to beers.

The impact of hops on beer aroma has been evaluated by many researchers. Hops produce an intense-smelling essential oil that comprises up to 3.0 % of the hop cone. The lupulin glands (**Figure 0-1c**), which are located on the leaves of the hop cones (**Figure 0-1b**), contain the essential oil as well as bitter-tasting hop acids. The composition of the hop essential oil is complex: 485 compounds have currently been identified in the literature [72, 79], and recent research suggests that up to 1000 might actually be present [79].

The elucidation of beer hop aroma is complicated, because the major constituents of hop oil are not transferred to the final beer; hence, the hop aroma that is detected in the beer differs significantly from the hop aroma that is detected by hand evaluation of the hops themselves [53, 75]. Various sample pretreatments (such as extraction, concentration, and clean-up steps) are necessary for the analysis of odorants in beer. Moreover, these odorants are only present in beer at trace levels, and complicated matrices (such as proteins, polyphenols, and fatty acids) can hinder their extraction for analysis. As a consequence, the precise details of the odorants comprising the beer hop aroma have remained unclear.

A detailed knowledge of these contributors is essential in order to manipulate and design beer hop aroma. The objective of the current research was to identify the odorants that comprise the beer hop aroma and their contributions to the aroma characteristics.

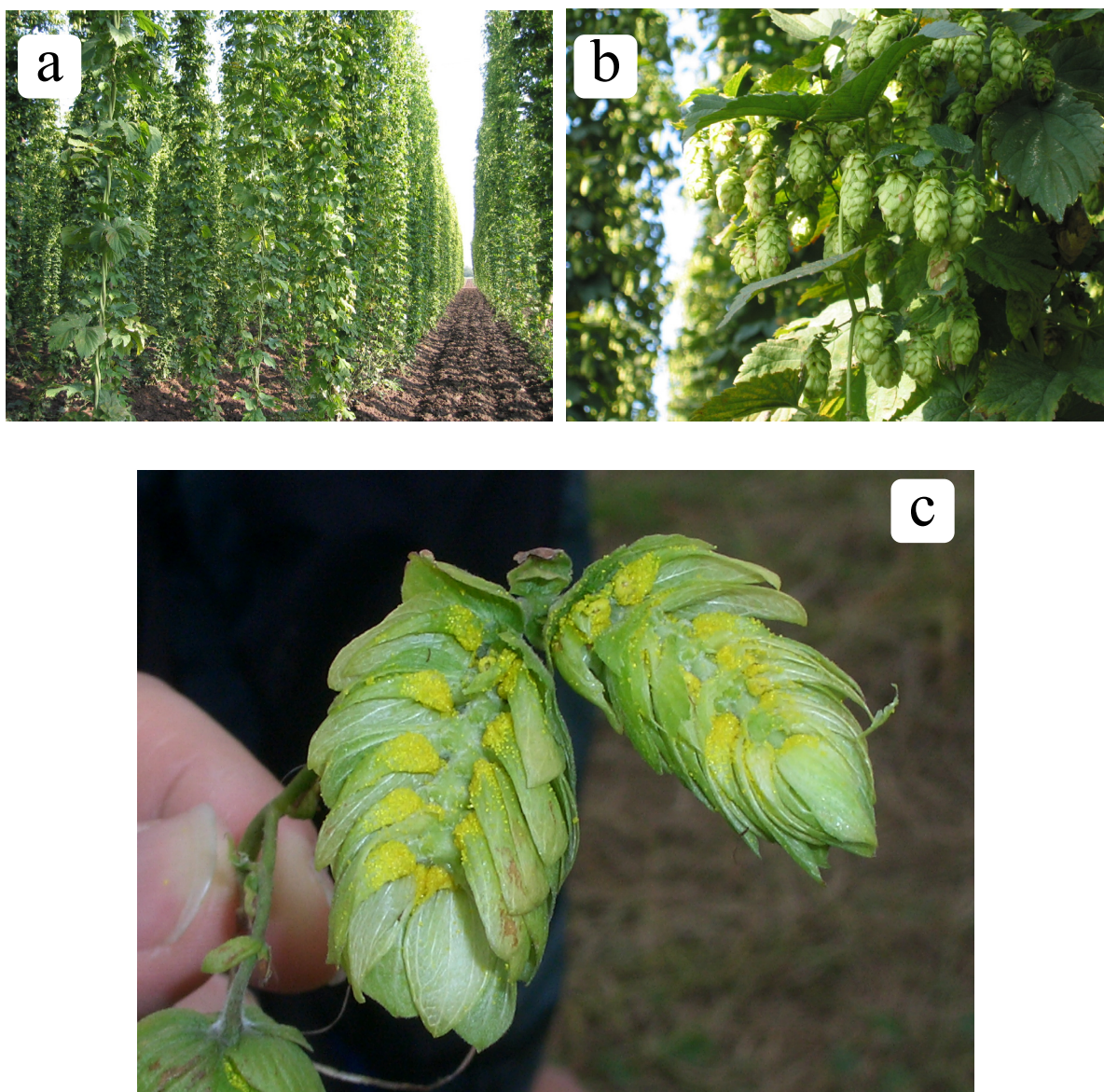


Figure 0-1. (a) Hops in a field. (b) Hop cones. (c) Lupulin glands (yellow) in the hop cone.

HOP CULTIVARS

Today, hops are grown as agricultural commodity in various parts of the world. The cultivated hops (*Humulus Lupulus L.*) are dioecious plant of the Cannabinaceae family, and now are almost exclusively used for beer brewing (**Figure 0-1**). Besides their brewing value, hops also have been traditionally used for medical purposes in pharmacopoeia, such as sedative properties.

Many different cultivars of the *Humulus Lupulus L.* species exist as shown in **Table 0-1**. The main commercial cultivars are grown in Europe, northwest region of the USA, Australia, New Zealand,

South Africa and China, while the largest growing countries are Germany and USA. In Japan, it is said that the cultivation of hops started in 1877 when Hokkaido Development Commissioner imported the hops from foreign country. Hops are cultivated under temperate climatic conditions, and the day-length requirements (hop requires a determinate amount of light during the growing season for flowering) restrict the possibility to cultivate hops commercially, with acceptable yields, to the latitude between 35° and 55° in both hemispheres. Regular supply of water during the growing season is required to assure good yield. Generally, water supply is provided by natural rainfall. In dry regions, such as the Yakima Valley in northwest USA, irrigation is necessary and better control of production and quality is achieved by the facilities.

Each cultivar has differences in composition of aroma and bitter substances [35, 52, 76]. Different cultivars provide various characteristic to beers, as described by Kaltner and Mitter [53], who observed that beers brewed with the cultivar Hallertauer have flowery and fruity notes and those with Styrian Golding have fruity, flowery, pine-resin note with high linalool content. New hop cultivars are being developed with considerably higher α -acid content up to 16 to 18%, such as Herkules, Apollo and Bravo.

Table 0-1. Cultivars and growing regions of hops (*Humulus Lupulus L.*). [8, 44, 70, 110]

Growing regions	Cultivars
USA	Ahtanum, Amarillo®, Apollo, Aquila, Banner, Bitter Gold, Bravo, Brewers Gold, Cascade, Centennial, Chelan, Chinook, Cluster, Columbus, Comet, Crystal, Eroica, Fuggle, Galena, Glacier, Golding US, Hallertauer, Horizon, Liberty, Magnum, Millennium, Mt. Hood, Newport, Northern Brewer, Nugget, Olympic, Palisade®, Perle, Saazer, Santiam, Satus®, Simcoe®, Spalter, Sterling, Strissel Spalt, Summit, Sun, Super Galena, Talisman, Tettnanger, Tillicum, Tomahawk®, Ultra, Vanguard, Warrior®, Willamette, Zeus
Germany	Brewers Gold, Columbus, Golden Princess, Hallertauer Magnum, Hallertauer Merkur, Hallertau Mittelfrüh, Hallertauer Taurus, Hallertauer Tradition, Herkules, Hersbrucker Pure, Hersbrucker Spät, Hüller Bitter, Northern Brewer, Nugget, Opal, Orion, Perle, Record, Relax, Saazer, Saphir, Smaragd, Spalter, Spalter Select, Tettnanger, Wye Target, Zeus
UK	Admiral, Boadicea, Bramling Cross, Brewers Gold, Bullion, Cobbs, Diva, Early Bird, Early Choice, Eastwell Golding, First Gold, Fuggle, Herald, Mathon, Northern Brewer, Omega, Phoenix, Pilgrim, Pilot, Pioneer, Progress, Sovereign, Whitbreads Golding, Wye Challenger, Wye Northdown, Wye Target, Yeoman
South Africa	Outeniqua, Southern Promise, Southern Star, Southern Brewer
Australia	Cascade, Cluster, Galaxy, Meteor, Millennium, Nova, Pride of Ringwood, Super Pride, Tasmanian Hallertau, Tasmanian Saazer, Topaz, Victoria, Willamette
New Zealand	Alpharoma, Green Bullet, Hallertau Aroma, Motueka, Nelson Sauvin, New Zealand Hallertauer, Pacifica, Pacific Gem, Pacific Jade, Pacific Sunrise, Pride of Ringwood,

	Riwaka, Southern Cross, Sticklebract, Super Alpha
Czech Republic	Agnus, Bor, Harmonie, Premiant, Rubin, Saazer, Sladek
France	Brewers Gold, Columbus, Hallertauer Magnum, Hallertauer Tradition, Nugget, Strisselspalter, Wye Target,
Poland	Iunga, Izabella, Limbus, Lomik, Lubelski, Lublin, Marynka, Nadwiślański, Oktawia, Sybilla, Zbyszko, Zula
Slovenia	Aurora (Super Styrian), Bobek, Hallertauer Magnum, Styrian Golding (Celeia)
Spain	Columbus, Hallertauer Magnum, Nugget, Perle
Republic of Serbia	Aroma, Bačka, Robusta
China	Tsingdao Flower, Marco Polo, Sapporo-1, Kirin Flower
Japan	Eastern Gold, Eastern Green, Fukuyutaka, Furano 18, Furano 6, Furano Ace, Furano Beta, Furano Laura, Furano Special, Golden Star, Kaikogane, Kitamidori, Little Star, Nanbuwase, SA-1, Shinsyu Wase, Sorachi Ace, Toyomidori

BREWING PROCESSES AND COMPONENTS IN DRIED HOP CONES

The water, hops, yeast, fermentable starch sources (such as barley or wheat, and adjunct including corn, rice, sugar, rye, sorghum, oats, etc.) are used as the basic ingredients of beer. The beer brewing process is composed mainly of mashing, sparging, boiling, fermentation, and packaging. Hops are added during or after the boiling process to provide a bitterness and characteristic aroma to beer.

Hundreds of components are contained in a hop cone. The major components present in dried hop cones are listed in **Table 0-2**. Intensely smelling essential oil, besides the bitter tasting hop acids, is contained in the lupulin glands, which located on the leaves of the hop cones (**Figure 0-1c**). In order to provide aroma to beer, hops are added at the end or after the boiling process to prevent the evaporation of the aroma components. The composition of hop essential oil is so complexed, with 485 compounds currently identified in the literature [72, 79] and recent research suggests that up to 1000 compounds may actually be present [79]. 50 – 80 % of essential oil is composed of hydrocarbons, and others are short chain acids, esters, thiols, and alcohols. Differences in aroma properties between hop cultivars can be attributed to variations in the composition of their essential oils [35]. The main hydrocarbons of hop oil are the terpenoids, and they had been examined as the main contributor to the hop aroma in beer [59].

The bitterness in beer that balances the sweetness from the malt derived from iso- α -acids.

Lupulin glands in hops include extremely hydrophobic precursors of bitter substances, α -acids and β -acids. α -acids consist mainly of co-humulone, normal-humulone and ad-humulone, and their ratio of concentration in α -acids is cultivar dependent. α -acids are isomerized into the water-soluble form; iso- α -acids during wort boiling process, while the β -acids are not isomerized and the majorities are lost in the brewing process due to their hydrophobicity. Therefore, iso- α -acids can be found in final beer products. For sufficient isomerization to provide bitterness, hops must be added at the beginning or earlier stage of boiling process. In addition to the characteristics, iso- α -acids are very surface active and thereby improve the foam stability of the beer. And bitter substances have an antibiotic effect that favors the activity of brewer's yeast over less desirable microorganisms [41, 43].

Table 0-2. Components present in dried hop cones. [26]

Major components	Concentration (% w/w)
Cellulose-lignins	40.0 – 50.0
Proteins	15.0
α -acids	2.0 – 17.0
β -acids	2.0 – 10.0
Water	8.0 – 12.0
Minerals	8.0 %
Polyphenols and tannins	3.0- 6.0
Lipids and fatty acids	1.0 – 5.0
Hop oil	0.5 – 3.0
Monosaccharides	2.0
Pectins	2.0
Amino acids	0.1

HOP CULTIVATION

The hop growing environments differ between countries, and each cultivar has differences in agronomic characteristics. Protection programmes using agrochemicals are necessary in all hop growing regions. Chemicals used for cultivating hops are shown in **Table 0-3**, and an effect of an agrochemical on the aroma components is investigated in the Chapter 3 and 4. The rootstocks of hops are perennial and can survive for many years; the useful recommended commercial life is about 12 to 15 years, while the above-ground parts of the plant die during the winter. The root system of

adult plants can extend more than 1.5 m in depth and more than 2 m laterally, therefore, rich and deep soils are required. At the beginning of spring, numerous shoots are produced from the buds of the upper part of the rootstock. New shoots have reached between 80 to 100 cm in length in the middle of spring, some are trained up string by the grower, and are tied to the fixed structures of poles and wires up to 5.5 – 8.0 metres high. Growth takes place between April and July (in the Northern Hemisphere), and is vigorous and fast, requiring large amounts of fertilizer. During July and August, the flowers of the female plants develop to form the hop cones (**Figure 0-1b**), which are rich in lupulin glands (**Figure 0-1c**) containing many different secondary metabolites including hop acids, oils and polyphenols.

During the growing season, some pests and diseases damage hops. The main pests that may attack hops are aphids (*Phorodon humuli*), red spider mites (*Tetranychus urticae*), and main diseases are downy mildew (*Pseudoperonospora humuli*) and powdery mildew (*Sphaerotheca humuli*), and Verticillium wilt (*Verticillium albo-atrum* and *V. dahliae*). Climate is considered as the major factor; a mild climate (average summer temperatures of 16 to 18 °C with frequent rainfall) favor the development of fungal disease, while hotter climates with low summer rainfall favor the red spider mite. These pests and diseases produce severe commercial deterioration (yield reduction, a deterioration of quality), or sometimes seriously stop cultivation.

Once hop cones have reached ripening, the crop is harvested and cones only are picked. Generally in the brewing, seedless female hops are used, because there exist some argue that oxidation of seed fat gives negative effect on beer foam. Hop cones at harvest have a moisture content of 75-80 % (w/w), and this needs to be reduced to less than 12 % (w/w) in order to prevent deterioration before processing, by hot air dryers (below 65°C) usually installed on the farms. Drying temperature, speed, and humidity have a major influence on the final content of aroma and hop acids. After the drying, cones are used for brewing as whole hop cones, or after the process into pellets or extracts.

Table 0-3. Chemicals used for cultivating hops [26]

Area of Application	
Downy mildew	Aluminiumfosetyl, Azoxystrobin, Copper, Dithianon, Dimethomorph, Folpet, Metalaxyl, Metalaxyl/ Folpet, Trifloxystrobin
Powdery mildew	Myclobutanil, Quinoxifen, Sulfur, Triadimenol, Trifloxystrobin

Alfalfa snout beetle/ Soil insects	Lambda-cyhalothrin
Hop aphid	Imidacloprid, Pymetrozin
Red spider mite	Abamectin, Fenpyroximat, Hexythiazox
Defoliants/ Weed control	Deiquat, Fluazifop-p-butyl, Haloxyfop, MCPA, Cinidon-ethyl

ANALYSIS OF HOP AROMA

Many researchers had studied hop aroma components, while not all character-impact odorants in hops have been identified [67].

In 1819, Hanin [39] firstly obtained oils from hops using steam distillation. In 1894 and 1895, Chapman identified 6 compounds of hop oil, including myrcene and humulene [14, 15]. He already noticed, in the early days of hop oil research, that myrcene and linalool elicit the typical scent of hops, and the oxidation products of hop oil were transferred into beer, while humulene which present in high amounts had no effect on the hop flavor [16].

In 1914, Rabak indicated that hydrocarbons, esters, alcohols, aldehydes, and organic acids are included in the hop oil [78]. The analysis of hop aromatic compound was revolutionized in the 1950's with the introduction of gas chromatography (GC). In 1957, Howard used the technique to separate 18 components of oil distilled from Fuggles hops [45]. The oil could further be split by chromatography on silica gel into hydrocarbon and oxygenated fractions [46]. When packed GC columns gave way to capillary columns, Buttery in 1966 was able to resolve around 100 components in USA, Australian and Japanese hop cultivars [12]. GC with flame-ionization detector (FID) has found universal use in analysis of hop aromatic compounds and in early studies permitted a crude characterization of different hop cultivars [62]. Subsequently, GC with flame photometric detection (FPD) revealed that hop oil can contain 20-30 sulphur compounds [77]. The later developed sievers chemiluminescence detector (SCD) offers several advantages over the FPD [51]. Modern studies have found GC coupled to mass spectrometry (GC-MS) as an indispensable tool, not only for the most sensitive quantification based on the monitoring of selected fragment ions [87] but for providing a vast amount of structural information on minor components.

In 1978, Tressl investigated hop aroma constituents in beer delivering a desirable hop aroma, using liquid-liquid extraction, liquid-solid chromatography with silica gel, aluminium oxide and GC-MS,

and identified more than 110 aroma constituents of German beer. Since then, a number of research groups have investigated the influence of hop cultivar, hop storage, hop processing and brewing conditions on the concentrations of hop oil compounds in beer, and attempted to relate these to the perceived hop aroma and flavor in the beer. The one feature which slowed progress is the great difficulties in obtaining purified extract to allow accurate quantification and proper evaluation. The odorants are present in beer at trace levels, and complicated matrices in beer interrupt the extraction of the odorants. Therefore various sample pretreatments (such as extraction, concentration and clean-up steps) are necessary for the analysis of hop terpenoids by GC-MS.

A range of extraction and concentration methods have been developed for the analysis of terpenoids, which include extraction with a conventional solvent [89], headspace apparatus [68], supercritical-fluid CO₂ extraction [21], column chromatography [48], and solid phase extraction [47]. Nickerson *et al.* reported the analysis with a cold finger trap that minimizes contact between condensed oil and steam [71]. Lam *et al.* [60] extracted aroma components with celite from 2 L of beer, and identified linalool, geraniol and β -citronellol as responsible for citrus and floral notes. De Keukeleire *et al.* [21] investigated differences in aromas between several cultivars of raw hops using supercritical-fluid CO₂ extraction, and identified myrcene, β -caryophyllene, α -humulene and β -farnesene as marker compounds. Lermusieau *et al.* [61] identified β -damascenone and linalool as odor-active constituents using XAD-2 resin chromatography. Recently, to resolve coelutions of compounds and separate volatile compounds more clearly, comprehensive two-dimensional gas chromatography (GC \times GC), which consists of two columns of different polar phases with a cryogenic modulator at the interface, and connected to time-of-flight mass spectrometry (TOFMS) was introduced to the analysis of hop oil components [32].

Many researchers made efforts to figure out the components comprising the characteristics of beer hop aroma. "Noble hop aroma" is a particularly desirable character in beer and is a term commonly used in the literature. This character is usually associated with the use of traditional aroma cultivars from Europe such as Hallertauer Hersbrucker and Saazer (**Table 0-1**) [22, 80]. The aroma description of this "noble" character is poorly defined, but is often described as herbal or spicy [80]. Oxidation and hydrolysis products of sesquiterpenoids have been associated with noble and spicy hop characters in beer [22, 34, 36, 80]. Good correlations between increasing concentrations of humulene epoxides and these hop characters have been demonstrated [75]. However, the importance of these oxidation compounds for imparting hoppy aroma remains controversial [34, 36, 48, 75, 80], because the compounds so far identified have concentrations below their detection thresholds, and their aroma characteristics do not correspond to the desired spicy or noble hop aroma [22].

As above described, the majority of past research on the character-impact odorants in hops and

beers has used instrumental data only, and focused on the identification of new volatile compounds, but neglected the evaluation of their respective aroma characteristic and contribution. The response of a physical GC detector (e.g., FID or MS) is not representative of odor activity [31], because the odor thresholds and odor intensities of volatile compounds vary considerably between compounds [13, 23], therefore, the most abundant compound in a chromatogram may not be the most important odorant. The impact of a compound on hoppy aroma must be evaluated using human assessors. Now it is well-accepted that only a limited number of volatiles actually do have an impact on the overall aroma of material [86].

In 1966, the first systematic study on the aroma contribution of individual hop volatiles was published by Guadagni *et al.* [38]. The authors calculated the aroma unit on the basis of the concentrations of major hop oil volatiles and their odor thresholds (aroma unit is expressed as the concentration of the compound divided by the difference threshold value [9]). The results confirmed, in particular, the importance of myrcene for the aroma of hops. A more sophisticated procedure for the identification of odor-active compounds is gas chromatography-olfactometry (GC-O). A valuable tool for identifying character-impact odorants is GC-O, where human assessors are used to detect and evaluate volatile compounds as they elute from a column following a GC separation [23].

The mainly used methods as GC-O are known as the aroma extract dilution analysis (AEDA) [85, 86] and CharmAnalysis [2, 3]. The major advantage of these techniques is that potent odorous compounds, often present in concentrations below the detection limits of conventional GC detectors, are nonetheless detected by causing a response in the assessor's nose at the sniffing port. In both procedures, an aroma extracts obtained from the sample is serially diluted, and each dilution is analyzed by GC-O [37]. In the case of AEDA, the result is expressed as flavor dilution (FD) factor, which is the ratio of the concentration of the odorant in the initial extract to its concentration in the most dilute extract in which the odor is still detectable by GC-O. In CharmAnalysis, a dilution series is prepared and each dilution is assessed by GC-O until no odors are perceived [2, 4]. Then analysis software constructs chromatographic peaks, and the peak areas were integrated to yield the Charm values as the sensory intensity of odorants. Both FD factor and Charm value are relative measures and are proportional to the aroma unit of the compound in air [4, 37]. The primary difference between two methods is that CharmAnalysis measures the dilution value over entire time the compounds elute, while AEDA simply determines the maximum dilution value detected [3]. Furthermore CharmAnalysis allows aroma components to be carried on air flowing at 30 ml/min [5], the flow of the odorants does not stay at the sniff port, and the boundaries between the aroma components are clearly defined.

Steinhaus *et al.* identified (2*E*)-*trans*-4,5-epoxy-2-decenal, (*R*)-linalool and myrcene as the most

potent odorants by application of AEDA on the volatile fraction isolated from a hop cultivar (Spalter Select) [86, 89]. However, the hop aromas as found in beer differs significantly from those found in hop cones themselves; the aroma qualities of hop cones are not reflected to the beer hop aroma. The impact of these hop aroma compounds on the flavor of a hopped Pilsner-type beer was later studied by Fritsch and Schieberle [33] using AEDA, aroma units and aroma simulation. Based on the calculation of odor activity values for compounds detected in hops as well as in the beer prepared thereof, it was shown that (*R*)-linalool was the only hop-derived compound still present in sufficient amounts to meet its odor threshold in the hopped beer. Only a few odorants that comprise the beer hop aroma are reported. The contributors to characteristics derived from cultivars and usage of hops still remain unclear.

In the current study, the author examined the odorants that comprise beer hop aroma and their contributions, in order to establish characteristic aroma in beer. The contents and behavior of terpenoids during the brewing processes, which are the main components of hop oil, are studied in the Chapter 1. The hop-derived potent odorants that persist even after fermentation and comprise hop aroma characteristics of beer are investigated in the Chapter 2. The contributions of the thiols, identified in the Chapter 2, are discussed in the Chapter 3 and 4. The contents of esters and terpenoids in beer, which were identified in the Chapter 2, and their contributions to the aroma of beer hopped with aged hops are examined in the Chapter 5.

Chapter 1

Analysis of Hop-Derived Terpenoids in Beer and Evaluation of Their Behavior Using the Stir Bar-Sorptive Extraction Method with GC-MS

1-1. INTRODUCTION

In order to control and design beer hop aroma, detailed knowledge of its components are required. The concentrations of terpenoids, such as myrcene, humulene, caryophyllene, have been the focus of several previous reports on hop aroma, because terpenoids in beers derive only from hops, and are the main components of hop oils.

In the previous studies, raw hops or beers with rich hop aromas were used in order to extract enough content of the hop-derived terpenoids for analysis [21, 36, 48, 60, 61, 89]. However, the aroma qualities of hop cones are not reflected in the beer hop aroma. These methods using large sample volumes were labor intensive, making them unsuitable for frequent or wide-ranging analyses. Furthermore, the trace levels of compounds still could not be detect using these methods. Thus, for the frequent analysis and investigation of beers with low-intensity hop aromas, a more sensitive analytical method with less effort is required.

Baltussen *et al.* [7] described a new extraction technique, known as the stir bar-sorptive extraction (SBSE) method, which is based on the partition coefficient between polydimethylsiloxane (PDMS) and water. This approach uses magnetic stir bars coated with 50–300 μL of PDMS and is sensitive and easy to use. Additional studies have evaluated this method for detect and quantify with trace volatiles in beers [19, 73], and with malt whisky [24]. In this chapter, the author used the SBSE approach to examine the behavior of terpenoids during the boiling process, the terpenoid contents of beers and their association with sensory characteristics.

1-2. MATERIALS AND METHODS

1-2-1. Reagents. Linalool, geraniol, myrcene, caryophyllene, α -humulene, β -damascenone and β -damascone were purchased from Fluka (Steinheim, Switzerland). β -citronellol and *cis*-3-hepten-1-ol were obtained from Sigma-Aldrich (St. Louis, MO) and Avocado Research Chemicals Ltd. (Lancashire, UK), respectively. Eudesmol, β -farnesene and synthesized humulene epoxide were purchased from Wako (Osaka, Japan). All reagents were analytical grade.

1-2-2. Preparation of Volatiles by Liquid Extraction. A 350-ml sample of beer containing 5 μ g of *cis*-3-hepten-1-ol (internal standard) was extracted with 150 ml of dichloromethane for 3 h at room temperature. The dichloromethane layer was then separated and dried over anhydrous sodium sulfate for 30 min. The extract was concentrated to approximately 1 ml at 750 hPa using a rotary evaporator at 40 °C.

1-2-3. GC-MS Conditions for the Liquid-Extraction Method. Separation of the extract was performed with an Agilent 6890 gas chromatograph coupled to a MSD5973N quadrupole mass spectrometer (Agilent Technologies, CA) equipped with a DB-WAX capillary column (60 m length \times 0.25 mm i.d.; film thickness = 0.25 μ m; Agilent Technologies) using pulsed splitless injection with helium carrier gas (1 ml/min). The inlet temperature was set at 250 °C, and the oven temperature was programmed from 40 °C (held for 5 min) up to 240 °C (held for 20 min) at a rate of 3 °C/min. A 1- μ L sample of concentrated volatile was injected into the GC-MS apparatus, which was set up to detect ions with a mass-to-charge ratio (m/z) of between 30 and 350, and operated in the electron-impact mode at 70 eV. All compounds were identified based on their mass spectra and retention time by comparison with authentic compounds.

1-2-4. Preparation of Volatiles using the SBSE Method. Stir bars (length = 20 mm) coated with 47 μ L of PDMS (Twister; Gerstel, Mulheim a/d Ruhr, Germany) were conditioned for 1 h at 300 °C in a stream of helium gas before use. β -damascone was added to the beer or wort sample at a final concentration of 0.1 μ g/L as an internal standard. A 30 ml of sample diluted with four volumes of distilled water was transferred into a vial and a PDMS-coated stir bar was added. After the vial was capped, the PDMS-coated bar was stirred in a water bath set at 40 °C for 2 h in order to extract the aroma substances. The stir bar was then withdrawn from the vial and washed with distilled water. After drying with a lint-free tissue, the stir bar was thermally desorbed into a GC-MS system via a thermal desorption unit (TDU; Gerstel) and a programmable temperature-vaporization inlet (CIS4;

Gerstel). Quantification was carried out in the selected ion monitoring (SIM) mode at the following m/z values: 69 (geraniol), 80 (α -humulene), 85 (humulenol II), 93 (linalool, myrcene, β -caryophyllene and β -farnesene), 123 (β -citronellol and humulene epoxide I), 149 (β -eudesmol), 177 (β -damascenone; internal standard) and 190 (β -damascenone).

1-2-5. Thermal Desorption GC-MS Conditions. Thermal desorption of the trapped aroma substances from the PDMS-coated stir bar was carried out in the TDU (**Figure 1-1**; Gerstel), which was programmed from 25 °C (held for 0 min) up to 240 °C (held for 5 min) at a rate of 2.5 °C/s in a splitless mode. The desorbed substances from the TDU were cryofocused in the CIS4 inlet at –100 °C using liquid nitrogen. The CIS4 inlet program for injecting the substances into the GC-MS column was started concomitantly with the initiation of the GC-MS program. The CIS4 inlet was programmed from –100 °C (held for 0 min) up to 240 °C (held for 5 min) at a rate of 2 °C/s in a splitless mode. The GC-MS conditions, with the exception of the inlet, were similar to those used in the preparation of volatiles by liquid extraction.



Figure 1-1. The GC-MS system with thermal desorption unit (TDU; Gerstel).

1-2-6. Brewing Processes. To investigate the terpenoid contents of different hop cultivars, Saaz (4.0 % α -acid pellets; Czech Republic), Tettnang (7.0 % α -acid pellets; Germany) and Hersbrucker (4.7 % α -acid pellets; Germany) were brewed in 3,000 L volumes. During each brewing, 67 % of the

total hops (based on the α -acid contents) was added at the beginning of the boiling process and the remaining 33 % was added 15 min before the end of this stage.

An additional 3000 L brew was performed to evaluate the behavior of terpenoids during and after boiling. Hersbrucker (4.7 % α -acid pellets) was added at the beginning of wort boiling and the behavior of the terpenoids was traced during the process.

1-2-7. Sensory Analysis. Flavor-profile analyses of Saaz, Tettnang and Hersbrucker beers were performed using a modified version of the Lermusieau [61] and Engel [28] methods. A panel comprising 19 trained individuals was asked to select from a list of attributes (hop pellet-like, resinous, green, floral, citrus, estery, muscat-like or spicy) after tasting, in order to describe the character of the hop aroma. The samples were served in a random order to each panelist.

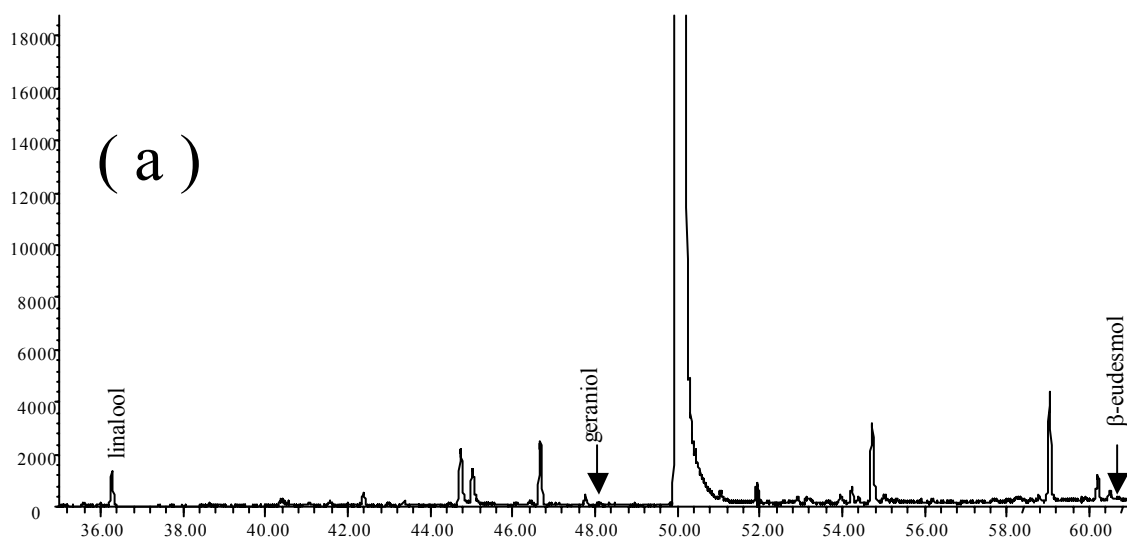
1-3. RESULTS AND DISCUSSION

1-3-1. SBSE Method. The author measured the amounts of terpenoids in Japanese commercial beers using both the conventional method (extraction with dichloromethane) employed in previous studies and the novel SBSE method. The amounts of compounds present were calculated from the ratio of the area of each sample to that of an internal standard compound. **Table 1-1** shows the amounts of terpenoids detected using the two different analytical methods in beers A and B, which were Japanese commercial beers with a poor and rich aroma, respectively. Only three compounds (linalool, geraniol and β -eudesmol) were detected by the conventional dichloromethane extraction. This was presumably due to the interference of the high matrix content (including proteins, amino acids and polyphenols) in the beers. By contrast, the SBSE method identified several additional substances. **Figure 1-2** shows selected ion m/z 93 chromatograms for general terpenoids. **Figure 1-3** shows m/z 190 chromatograms for β -damascenone from a Japanese commercial beer prepared using the dichloromethane extraction and SBSE methods. These results clearly illustrate that the SBSE method identified several substances that were not detected by the conventional dichloromethane extraction. Among the hundreds of substances that can potentially be detected by the SBSE method, the author focused on those introduced as potent odorants or marker compounds to the beers (as discussed above).

Table 1-1. Concentration of terpenoids ($\mu\text{g/L}$) in Japanese commercial beers^a.

	SBSE method		Extraction with dichloromethane	
	Japanese beer	Japanese beer	Japanese beer	Japanese beer
	A	B	A	B
Linalool	1.31	4.57	1.16	4.97
Geraniol	2.44	3.49	2.25	3.45
β -citronellol	3.01	4.26	ND	ND
Myrcene	0.47	0.34	ND	ND
β -caryophyllene	0.19	ND	ND	ND
α -humulene	0.73	0.23	ND	ND
Humulene epoxide I	0.10	0.53	ND	ND
β -eudesmol	1.27	0.43	1.07	0.50
β -farnesene	0.36	0.54	ND	ND
β -damascenone	1.38	2.23	ND	ND

^aTerpenoids were extracted by dichloromethane from 350 ml beer and by the SBSE method from 6 ml of beer. ND, not detected.



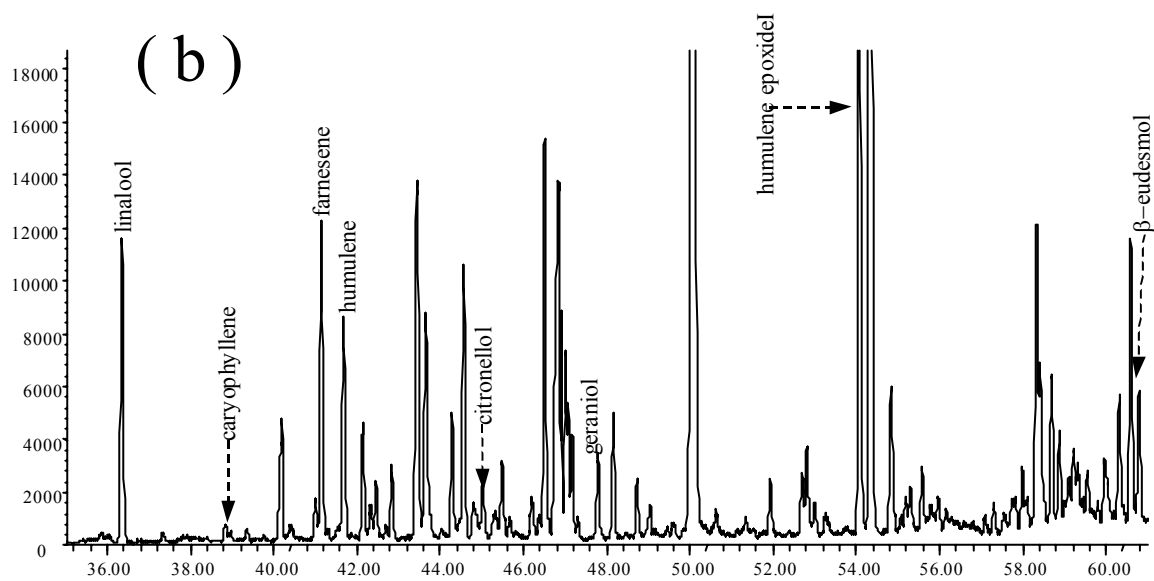


Figure 1-2. Selected ion chromatogram (m/z 93) of volatiles prepared by dichloromethane extraction (a) and by the SBSE method (b) from Japanese commercial beer B.

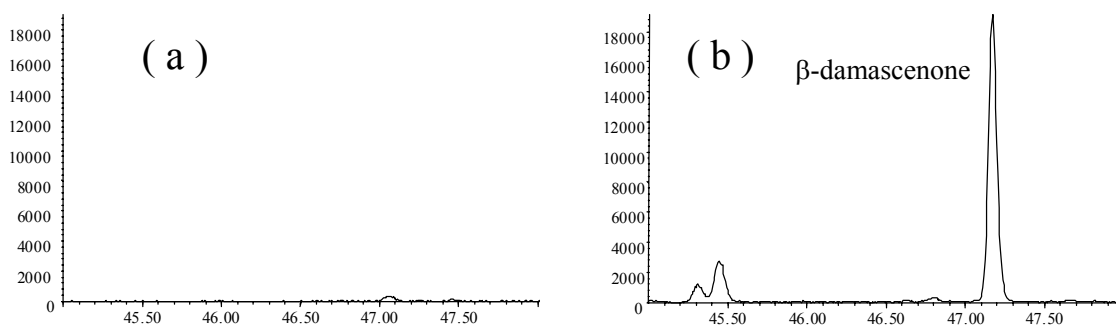


Figure 1-3. Selected ion chromatogram (m/z 190) of volatiles prepared by dichloromethane extraction (a) and by the SBSE method (b) from Japanese commercial beer B.

The theory behind the SBSE method is straightforward [7]. A PDMS-coated stir bar is introduced into the beer or wort sample and the extraction occurs during the stirring process, as the hydrophobic substances are absorbed into the PDMS. The logarithm of the PDMS–water partition coefficient is roughly equivalent to the logarithm octanol–water partition coefficient (LogKow); Kow is a physical parameter that is commonly used to describe the hydrophilic or hydrophobic properties of chemicals [64] and logarithm of that (LogKow) is generally used to characterize its value. The LogKow values of the terpenoids calculated by SRC-KOWWIN software (Syracuse Research, Syracuse, NY) are shown in **Table 1-2**. A high LogKow value indicates high hydrophobicity and most likely a high recovery by the PDMS-coated stir bar. The PDMS–water partition attains equilibrium rapidly at higher temperatures; my observations indicated that a temperature of 40 °C was appropriate for a 2-hr extraction. The LogKow values of the terpenoids ranged between 3.38 and 7.10. β -damascone was selected as the internal standard compound as its LogKow value (4.42) fell within this range, and strong correlations between the areas of β -damascenone and each terpenoid were observed.

Table 1-2 shows the coefficients of variation (CVs), detection limits, and correlations (r^2) between the internal standard ratio and the observed amounts according to the SBSE method. This technique showed high sensitivity coupled with low detection limits (0.001 to 0.28 $\mu\text{g/L}$), low CVs (less than 10 %), and high correlations between the internal standard compound and the sample concentrations (greater than 0.99).

1-3-2. Behavior of Hop Terpenoids throughout Wort Boiling Process. The simplicity of the SBSE method, which requires small sample volumes and is less labor intensive, allowed us to trace the behavior of hop terpenoids throughout wort boiling process (**Figure 1-4**). Caryophyllene, humulene and β -farnesene are generally present at high concentrations in hop pellets, but at relatively low concentrations during the boiling process. These facts confirm their poor solubility in wort. β -citronellol was not detected in the wort samples (data not shown), as it was transformed from geraniol during fermentation [54].

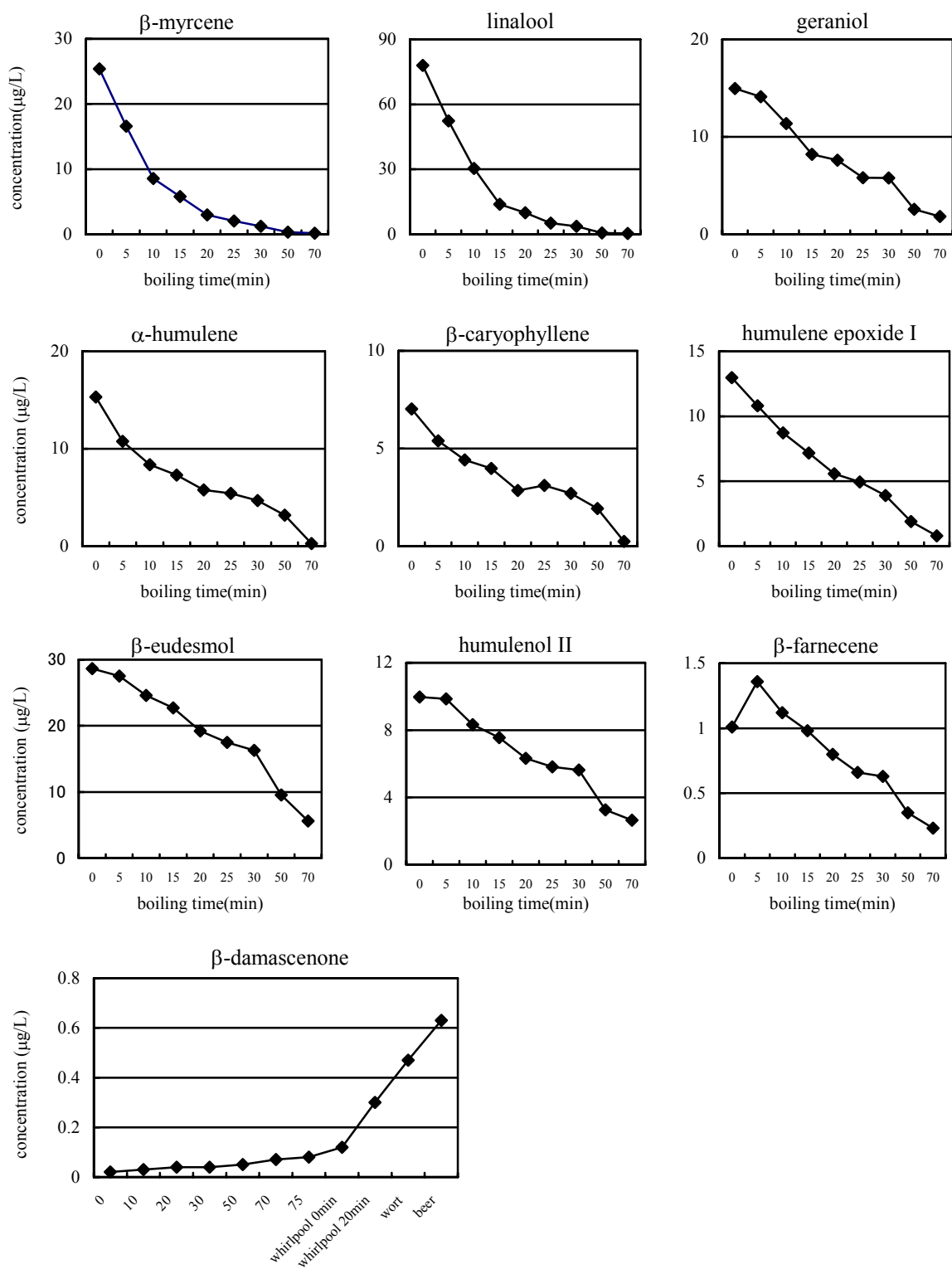


Figure 1-4. Behavior of hop-derived terpenoids during wort boiling.

Two distinct patterns of decrease were detected among the terpenoids. The first was seen in myrcene and linalool, the levels of which fell rapidly during the boiling process in a pattern corresponding to a quadratic curve. This was partly due to their low boiling points (**Table 1-2**), which were reflected in various aspects of their chemical structure (for example, the number of carbons, the types of functional groups and the positions of the double bonds). Hops must therefore be added towards the end or after the boiling process in order to retain higher concentrations of these terpenoids. The second pattern was observed in β -eudesmol, humulene, humulene epoxide I, β -farnesene, caryophyllene and geraniol, all of which have higher boiling points (**Table 1-2**); the concentrations of these components decreased gently and linearly throughout the boiling process. These two distinct patterns supported my observation that the hop aroma characters of beers depend upon the time at which the hops are added.

Table 1-2. CVs, detection limits and r^2 values for terpenoids analyzed by the SBSE method.

	Log Kow	CV (%) ^a	Detection limit ($\mu\text{g/L}$) ^b	r^2 ^c	Boiling point ($^{\circ}\text{C}$) at 760 mmHg
Linalool	3.38	4.3	0.049	0.997	194
Geraniol	3.47	5.7	0.099	0.997	229
β -citronellol	3.56	6.8	0.278	0.999	–
Myrcene	4.88	7.4	0.001	0.999	167
β -caryophyllene	6.30	8.2	0.031	0.999	262
α -humulene	6.95	4.2	0.035	0.999	266
Humulene epoxide I	5.55	2.1	0.044	0.994	–
β -eudesmol	4.88	5.5	0.013	1.000	–
β -farnesene	7.10	6.7	0.023	1.000	260
β -damascenone	4.21	2.0	0.019	1.000	–
β -damascenone (IS)	4.42	–	–	–	–

^a CVs were calculated from concentrations obtained by injections between 6 days. ^b The concentration when the signal/ noise was 3. ^c The correlation between the internal standard and the observed concentrations. IS, internal standard.

Interestingly, an alternative pattern was seen in β -damascenone, the concentration of which increased after boiling. Isoe *et al.* [49] suggested that β -damascenone could be formed by the acid-catalyzed conversion of polyols (enyne diols or allene triols) resulting from enzymatic transformations of the carotenoid neoxanthin. Chevance *et al.* [17] also proposed that

β -damascenone was released by the acidic hydrolysis of glycosides during the aging of beers. Conversely, Kotseridis *et al.* [56] reported increased β -damascenone levels in wine after heat treatment. The author also observed β -damascenone formation both during and after boiling; however, the β -damascenone that was formed during this process evaporated immediately, so the increase could only be detected after boiling.

Table 1-3. Concentration of terpenoids ($\mu\text{g/L}$) in beers brewed using different hop cultivars.

	Saaz	Tettnang	Hersbrucker	Thresholds ($\mu\text{g/L}$)	Characteristics
Linalool	4.8	4.0	10.5	27 ^a	Floral, citrus ^g
Geraniol	3.5	3.4	3.8	36 ^a	Floral, citrus ^g , rose-like ^b
β -citronellol	3.0	2.0	2.2	11 (in water) ^b	Rose-like ^b , floral, citrus ^g
Myrcene	0.7	0.4	1.1	30–200 ^c	Resinous ^j
β -caryophyllene	0.3	0.2	0.1	450 ^c	Clove, turpentine ^b
α -humulene	0.3	0.7	0.2	450 ^c	–
Humulene epoxide I	5.5	5.3	7.1	10 (in water) ^d	Hay-like ^g
Humulenol II	4.1	3.5	6.5	2500 ^a	Sagebrush-like ^g
β -eudesmol	0.6	0.6	17.5	> 10000 ^e	Contained in spicy fraction ^e
β -farnesene	1.4	1.4	1.6	550 ^c	–
β -damascenone	0.9	0.8	0.6	0.02–0.09 ^f (in water)	Apple, peach ^h , fruity ^f

The values are shown relative to the threshold in: beer [11]^a, water [11]^b, beer [88]^c, water [103]^d, beer [48]^e, beer [17]^f, beer [60]^g, beer [58]^h, and beer [84]^j.

1-3-3. Differences of Beer Terpenoid Contents between Beers Hopped with Different Cultivars.

The author studied the differences of beer terpenoid contents between the hop cultivars using the SBSE method. **Table 1-3** shows the amounts of terpenoids in beers brewed using different cultivars of hops. Hersbrucker beer contained greater amounts of linalool, myrcene, humulene epoxide I, humulenol II and β -eudesmol compared with the other two cultivars, even when the α -acid contents were taken into

consideration. Humulene epoxides are known to reflect deterioration of the hops [24, 56]. The author therefore used fresh cold-stored hops in which no such changes were observed before the brewing process. It was believed that the high concentrations detected in Hersbrucker beer reflected high concentrations in the original hop pellets. In particular, the β -eudesmol concentration in Hersbrucker was extremely high, which is a characteristic of this hop cultivar [69]. The hop aroma character of beer is reported to depend upon the hop cultivar that is used. This observation was partially supported by the differences in hop oil composition described here.

1-3-4. Relationship between Terpenoid Contents and Sensory Analysis. Finally, the author investigated the relationship between the terpenoid content and sensory analysis. **Table 1-4** shows the results of the sensory evaluation of beers that were brewed using different hop cultivars; the data indicate the numbers of panelists who selected each attribute. **Table 1-3** shows the concentrations of terpenoids in beers brewed using different hop cultivars, along with the detection thresholds and characteristics of each terpenoid.

The number of panelists who selected the terms ‘floral’, ‘hop pellet-like’ and ‘green’ to describe Hersbrucker beer was higher than for the other two types of beer. Previous reports [48, 61] indicated that the sesquiterpenoid fraction (containing eudesmol, humulene epoxides and humulenol II) contributed to the spicy hop character of Hersbrucker beer. Another study [60] suggested that linalool, geraniol and β -citronellol contributed to the citrus and floral notes. In addition, myrcene, β -caryophyllene, α -humulene and β -farnesene have been identified as marker compounds between the different hop cultivars [21]. In this chapter, all of the terpenoids measured were lower than the threshold values. Among these, the floral and hop pellet-like characteristics might partly correspond to increased amounts of linalool and humulene epoxide I, respectively, because the concentrations of these terpenoids were closest to the threshold values. However, the ‘spicy’ character was not reflected by the concentrations of sesquiterpenoid. The number of panelists who selected the term ‘resinous’ to describe Saaz beer was larger than those for the other two beers. The concentration of myrcene, which has a resinous character, was far lower than the threshold value and did not contribute independently to the character. Thus, other resinous compounds should be investigated in future studies. Not all of the sensory characteristics could be explained from results, particularly in terms of the higher threshold substances, and relatively small amounts of substances might have contributed to these qualities.

In conclusion, changes in the concentrations of hop-derived terpenoids during the boiling process showed three distinct patterns. Most terpenoid concentrations decreased during boiling as a result of

evaporation and the patterns were either linear or followed a rapid quadratic curve depending upon the boiling point. By contrast, the β -damascenone concentration increased slowly during the boiling process and rose dramatically thereafter during the relatively cool whirlpool-processing step. Some associations between the terpenoid contents and sensory analyses of beers were detected, but it was difficult to explain the relationships clearly, particularly in terms of the higher threshold substances. Further investigations for the odorants comprising hop aroma characteristics are described in the Chapter 2.

Table 1-4. Sensory evaluation of beers brewed using different hop cultivars ^a.

	Saaz	Tettnang	Hersbrucker
Hop pellet-like	2	3	5
Resinous	6	2	3
Green	5	2	7
Floral	4	5	9
Citrus	3	2	3
Estery	4	5	4
Muscat-like	1	1	3
Spicy	2	1	1

^a The data in the table indicate the number of panelists (n=19) who selected each attribute.

Chapter 2

Comparison of the Odor-Active Compounds in Unhopped Beer and Beers Hopped with Different Hop Cultivars

2-1. INTRODUCTION

The impact of hops on beer flavor had been evaluated in many researches. The majority of past researches have used quantitative analysis employing GC-FID or GC-MS to assess the impact of hops on beer flavor.

In the Chapter 1, hop-derived terpenoids were evaluated by using the highly sensitive and quantitative SBSE method with GC-MS, because the aroma in hop cones consists mainly of terpenoids, such as myrcene, humulene, caryophyllene. Using this technique, the author showed that most hop-derived terpenoids were lost during fermentation, possibly due to their highly hydrophobic properties. Hydrophobic or high-molecular weight substances are absorbed during the hot/ cold break or by yeast [53]. Some components are metabolized, particularly through ester hydrolysis and esterification by yeast [53, 54]. Moreover, it was revealed that these terpenoids have high threshold values and, thus, make relatively little contribution to the hop aroma. Unidentified substances or the odorants present at trace levels, therefore, may comprise the beer hop aroma in beer. The use of GC-O techniques, such as AEDA [33, 89, 105] and CharmAnalysisTM [1] in combination with quantitative analysis is necessary to determine the odor-active components in beer.

In this chapter, to reveal the hop-derived odor-active components that persist even after fermentation and comprise the hop aromas in beers, the comparison of beers hopped with different cultivars and unhopped beer was performed using CharmAnalysisTM in combination with quantification and the sensory evaluation.

2-2. MATERIALS AND METHODS

2-2-1. Reagents. Ethyl 2-methylpropanoate (Chemical Abstracts Service [CAS] No. 97-62-1), *o*-aminoacetophenone (CAS No. 551-93-9), 2-methyl-3-furanthiol (CAS No. 28588-74-1), 3-methylindole (CAS No. 83-34-1), 2-methoxy-4-vinylphenol (CAS No. 7786-61-0), decanoic acid (CAS No. 334-48-5), ethyl butyrate (CAS No. 105-54-4), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (CAS No. 3658-77-3), 2-furanmethanethiol (CAS No. 98-02-2), γ -nonalactone (CAS No. 104-61-0), *o*-methoxyphenol (CAS No. 90-05-1), hexanoic acid (CAS No. 142-62-1), 1-hexanol (CAS No. 111-27-3), indole (CAS No. 120-72-9), isoamyl acetate (CAS No. 123-92-2), isoamyl alcohol (CAS No. 123-51-3), 2-methylpropanoic acid (CAS No. 79-31-2), 3-hydroxy-2-methyl-4-pyrone (CAS No. 118-71-8), 3-methylthiopropionaldehyde (CAS No. 3268-49-3), 3-methylthiopropanol (CAS No. 505-10-2), octanoic acid (CAS No. 124-07-2), ethyl hexanoate (CAS No. 123-66-0), *n*-butyric acid (CAS No. 107-92-6), 3-methylbutanoic acid (CAS No. 503-74-2), β -phenylethyl alcohol (CAS No. 60-12-8) and vanillin (CAS No. 121-33-5) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). β -ionone (CAS No. 79-77-6), (*Z*)-3-hexen-1-ol (CAS No. 928-96-1), 3-methyl-2-butenal (CAS No. 107-86-8), (*Z*)-3-hexenal (CAS No. 6789-80-6), 2,3-butanedione (CAS No. 431-03-8), β -damascenone (CAS No. 23696-85-7) and (*E,Z*)-2,6-nonadienal (CAS No. 557-48-2) were purchased from Sigma-Aldrich (Missouri, USA). (+/-)-ethyl 2-methylbutanoate (CAS No. 7452-79-1), 2-phenylethyl 3-methylbutanoate (CAS No. 140-26-1), 4-(4-hydroxyphenyl)-2-butanone (CAS No. 5471-51-2) and (*Z*)-3-hexenoic acid (CAS No. 4219-24-3) were purchased from Acros Organics (New Jersey, USA). (*Z*)-1,5-octadien-3-one (CAS No. 65767-22-8), 4-mercapto-4-methylpentan-2-one (CAS No. 19872-52-7), 2-acetyl-1-pyrroline (CAS No. 85213-22-5), 2-mercapto-3-methyl-1-butanol, 2-propionyl-1-pyrroline (CAS No. 133447-37-7) and 3-mercapto-2-methyl-1-butanol were obtained from San-Ei Gen FFI, Inc. (Osaka, Japan). (*R/S*)-linalool (CAS No. 78-70-6), (*R*)-linalool (CAS No. 126-91-0), geraniol (CAS No. 106-24-1), β -damascone (CAS No. 23726-91-2), ethyl 3-methylbutanoate (CAS No. 108-64-5), ethyl 4-methylpentanoate (CAS No. 25415-67-2), (-)-borneol (CAS No. 464-45-9) and (*Z*)-3-hepten-1-ol (CAS No. 1708-81-2) were purchased from Fluka (Buchs, Switzerland). 3-mercaptohexan-1-ol (CAS No. 51755-83-0) was purchased from Avocado Research Chemicals Ltd. (Lancashire, UK). 1-hexanal (CAS No. 66-25-1) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 3-methyl-2-butene-1-thiol (CAS No. 5287-45-6) were obtained from Tokyo Chemical Industry CO., LTD. (Tokyo, Japan). *trans*-4,5-Epoxy-2(*E*)-decenal (CAS No. 134454-31-2) was purchased from Cayman Chemical Co. (Michigan, USA).

2-2-2. Brewing Processes. Saazer (5.6 % α -acid pellets; Czech Republic), Cascade (5.5 % α -acid pellets; USA) and Hersbrucker (3.8 % α -acid pellets; Germany) were used in the brewing processes. Beers hopped with different cultivars and unhopped beer were brewed independently at a 20-L volume scale. Where appropriate, 30 g of hops were added at the beginning of the boiling process and a further 60 g after the end of the process.

Static fermentations were carried out in 5 L stainless-steel tall tubes, modified from 2 L European Brewery Convention (EBC) tall tubes [25], which were equipped with precise pressure and temperature controls (**Figure 2-1**). The yeast used in the fermentations was washed twice (for 5 days each time) in unhopped wort. The washed yeast was pitched at a rate of 20×10^6 cells/ml into the wort. A 5-L sample of each wort (11.5 °P) was fermented at 12.0 °C for 6 days, under an inner-tank pressure of 0.05 MPa. Then any yeast that settled on the tank bottom was eliminated. Each beer was then allowed to mature at 12.0 °C for 5 days. The finished beer was obtained by cooling to -1 °C for 5 days and then centrifuging at 7000 rpm for 30 min.



Figure 2-1. The 5-L stainless-steel tall tubes for fermentation, equipped with the strict controller of pressure and temperature.

2-2-3. Isolation of the Volatiles for GC–O Analysis. A 2-L sample of each beer was extracted with 1 L dichloromethane by stirring gently, without making an emulsion, for 12 h at 4 °C. The dichloromethane and aqueous layers were separated, and the former dried over anhydrous sodium sulfate for 30 min. Each extract was then carefully concentrated to 10 ml using a Kuderna–Danish evaporative concentrator (**Figure 2-2**).

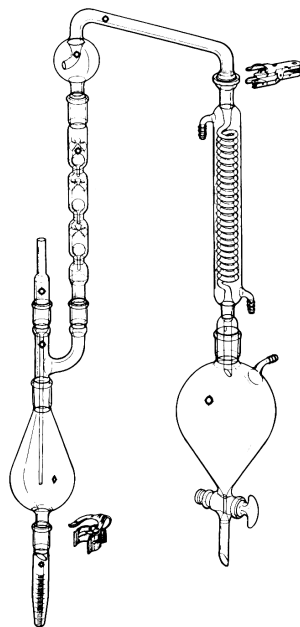


Figure 2-2. Kuderna–Danish evaporative concentrator.

2-2-4. GC–O Analysis. CharmAnalysisTM was conducted on an Agilent 6890 gas chromatograph (Agilent Technologies, CA), which was modified by DATU Inc. (Geneva, NY) and equipped with an DB-WAX capillary column (Agilent Technologies; length = 15 m; i.d. = 0.32 mm; film thickness = 0.25 μ m), using helium (1 ml/min) as a carrier gas. The rate of humidified air-flow into the sniff port was set at 30 ml/min at 60 °C. The inlet temperature was set at 225 °C in the splitless mode, and the oven temperature was programmed to rise from 40 to 230 °C (held for 20 min) at a rate of 6 °C/min. Samples of 1 μ L were injected into the testing apparatus.

The original odor extract of each beer was stepwise diluted with dichloromethane to 3ⁿ (where n = 0–3). A dilution series was analyzed for each extract, ranging from undiluted concentrate to a 1:27 dilution. Quantitative responses to the eluting aromas were generated using Charmware (DATU Inc.). A series of alkanes (C10–C32) was also analyzed using FID to establish the Kovats retention indices (RIs).

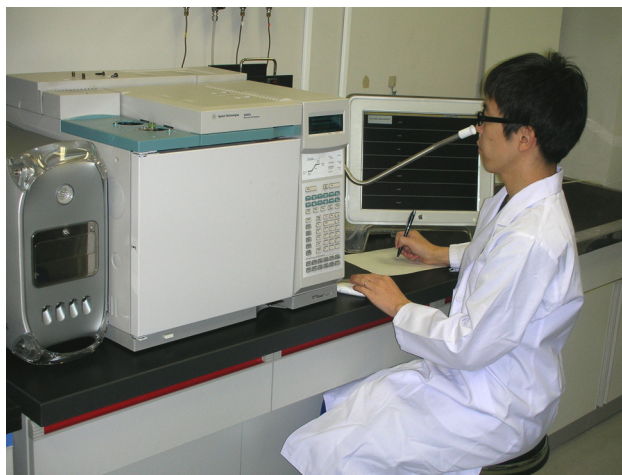


Figure 2-3. CharmAnalysis™ on Agilent 6890 gas chromatograph.

2-2-5. Sensory Evaluation. Flavor-profile analyses were performed for Saazer, Hersbrucker, and Cascade beers comparing to the unhopped beer by seven trained sensory panelists. The five attributes that best expressed the hop aroma characteristics were selected by tasting commercial and pilot test beers; these were identified as ‘green’, ‘citrus’, ‘floral’, ‘spicy’, and ‘muscat/ blackcurrant-like’. The responses to each description were shown to be consistent between seven trained sensory panelists using matching test [66]. The members of the sensory panel were then asked to evaluate the total hop aroma intensity and the intensity of the five odor attributes for the beers hopped with different cultivars by setting the intensity of each attribute for unhopped beer as control. The respective odor intensities were rated on the following scale (using 0.5-interval steps): 0 = not perceivable; 1 = weak; 2 = normal; 3 = strong; 4 = very strong. The characteristics between the cultivars were compared following the Scheffé’s method [83] by calculating the mean intensity value of the score and the paired t-test value for the characteristics.

2-2-6. Determination of Difference Threshold Values. The orthonasal difference threshold values of ethyl 2-methylpropanoate, (+/-)-ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 4-methylpentanoate, 4-mercapto-4-methylpentan-2-one (4MMP), (Z)-1,5-octadien-3-one, (Z)-3-hexen-1-ol, (R)-linalool, 3-mercaptohexan-1-ol (3MH), geraniol, β -ionone, 2-phenylethyl-3-methylbutanoate, and 4-(4-hydroxyphenyl)-2-butanone were determined using the method of American Society of Brewing Chemists (ASBC) [10, 94]. The threshold values were established by a triangle test using a series of six concentrations. An ethanol solution of the chemical was added to a light-tasting Japanese beer. During the test, the members of a panel comprising nine

trained individuals were asked to taste three samples and to identify the odd one out. The best estimate threshold was calculated for each assessor as the geometric mean of the highest concentration missed and the next highest concentration. The group threshold was calculated as the geometric mean of the best estimate thresholds of the assessors.

2-2-7. Identification of Odorants. Identifications of the hop-derived components were attempted by GC-MS and CharmAnalysisTM equipped with DB-WAX and DB-1 column comparing their RIs, mass spectra, and odor qualities with those of the authentic compounds.

The separation of each extract in GC-MS was performed with an Agilent 6890 gas chromatograph coupled to an Agilent MSD5973N quadrupole mass spectrometer equipped with a DB-WAX and a DB-1 capillary column (Agilent Technologies; length = 60 m; i.d. = 0.25 mm; film thickness = 0.25 μ m) respectively, using pulsed splitless injection with helium (1 ml/min) as a carrier gas. The inlet temperature was set at 250 °C, and the oven temperature was programmed to rise from 40 °C (held for 5 min) to 240 °C (held for 20 min) at a rate of 3°C/min. A 1- μ L sample of concentrated volatile was injected into the GC-MS apparatus, which was set up to detect ions with a mass-to-charge ratio (m/z) of 30–350, and was operated in the electron-impact mode at 70 eV.

2-2-8. Quantification of Volatiles. The quantification of linalool, geraniol, and β -ionone in wort and beer was carried out by the SBSE method using β -damascone as an internal standard, as described in the Chapter 1. The amounts of ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 1-hexanal, (*Z*)-3-hexen-1-ol, 2-phenylethyl 3-methylbutanoate and 4-(4-hydroxyphenyl)-2-butanone in wort and beer were measured by the liquid-extraction method with dichloromethane using (-)-borneol and (*Z*)-3-hepten-1-ol as an internal standard, as described in the Chapter 1. The data are shown as the mean values of duplicated analysis.

2-3. RESULTS AND DISCUSSION

Unhopped beer and strongly hopped beers, using approximately fivefold amount of hops than normal, were brewed to distinguish clearly the characteristics of the aromas and the compounds contributing to the characteristics. Each extract was carefully concentrated using a Kuderna–Danish evaporative concentrator, in order to reduce the loss of highly volatile odorants.

The odorants which were observed in this GC-O analysis are shown in **Table 2-1** along with the

identified components. Beer extracts contain numerous aroma components and matrices which derive from malts, hops, and the process of fermentation, thus many odorants are presented simultaneously during GC–O. In this study, a wide range of aromas comprising 83 odorants was detected since CharmAnalysis™ allows aroma components to be carried on air flowing at 30 ml/min [5], the flow of the odorants does not stay at the sniff port, and the boundaries between the aroma components are clearly defined. Chromatographic peaks for each extract are generated in CharmAnalysis™, and the peak areas were integrated to yield the Charm values [3] shown in **Table 2-1**.

Table 2-1. Charm value for the volatile fraction of unhopped, Saazer, Hersbrucker, and Cascade beers.

RI on DB-WAX	odor quality	Charm value				compounds
		unhopped	Saazer	Hersbrucker	Cascade	
962	solvent	0	32	58	24	
976	citrus	49	22	14	0	
996	diacetyl	211	36	372	238	2,3-butanedione ^d
1004	citrus, pineapple, sweet	348	1125	1582	1252	ethyl 2-methylpropanoate ^a
1048	solvent	2685	1016	1584	2132	
1050	citrus	1981	1368	1090	1664	ethyl butyrate ^a
1068	citrus, apple-like	114	952	1370	968	(+/-)-ethyl 2-methylbutanoate ^a
1084	citrus, sweet, apple-like	0	1601	765	564	ethyl 3-methylbutanoate ^a
1103	green, leafy	0	493	568	712	1-hexanal ^a
1111	almond, roasted	88	426	490	410	3-methyl-2-butene-1-thiol ^b
1129	solvent	1060	1202	711	1095	isoamyl acetate ^a
1148	green, leafy	72	1081	1111	1002	(Z)-3-hexenal ^a
1180	citrus, pineapple	0	1286	646	647	ethyl 4-methylpentanoate ^a
1190	resinous	0	247	618	205	myrcene ^a
1211	floral	1844	1936	1682	1830	isoamyl alcohol ^a
1235	citrus, estery	705	479	165	567	ethyl hexanoate ^a
1298	roasted meat, vitamin b	1744	1273	1059	1824	2-methyl-3-furanthiol ^d
1326	popcorn-like	728	1096	729	809	2-acetyl-1-pyrroline ^d
1338	fruity, catty, thiol-like	43	241	486	302	

1350	green, leafy	524	444	974	539	1-hexanol ^a
1363	muscat/ blackcurrant-like, fruity	0	612	479	1083	4-mercapto-4-methylpentan-2-one ^b
1373	green, metallic	41	1016	1073	450	(Z)-1,5-octadien-3-one ^b
1383	muscat-like, green	133	566	415	1186	(Z)-3-hexen-1-ol ^a
1413	cracker-like	284	89	8	26	2-propionyl-1-pyrroline ^d
1422	roasted meat	983	502	572	684	2-furanmethanethiol ^d
1424	solvent	799	738	680	883	
1430	potato, soy-sauce	1102	1029	416	214	3-methylthiopropionaldehyde ^d
1454	musty	207	224	213	251	
1495	solvent	1372	1150	1101	746	
1508	sweet	473	178	164	20	
1516	cracker-like	763	574	599	337	
1540	green	582	112	443	142	
1548	floral, citrus, terpenic	28	1066	1329	1011	(R/S)-linalool ^a
1561	cheesy	353	227	130	411	2-methylpropanoic acid ^c
1571	green, cucumber	0	178	370	237	(E,Z)-2,6-nonadienal ^a
1590	green, metallic	0	612	1304	871	
1600	roasted meat	536	641	394	311	
1614	cheesy	1368	938	714	738	n-butyric acid ^a
1647	meat, onion-like	94	0	122	223	2-mercapto-3-methyl-1-butanol ^d
1652	cheesy	1304	1330	1115	1197	3-methylbutanoic acid ^a
1682	fatty	218	750	653	633	
1704	potato, soy-sauce	1078	296	423	1131	3-methylthiopropanol ^c
1721	meat, onion-like	427	69	194	285	3-mercapto-2-methyl-1-butanol ^d
1730	cracker-like	735	924	15	787	
1789	citrus	1292	697	505	1386	β-damascenone ^a
1810	roasted, chocolate	778	988	644	781	
1825	fruity, catty, thiol-like	151	1165	1010	1226	3-mercaptohexan-1-ol ^b
1834	sweet	833	737	501	606	o-methoxyphenol ^c
1838	rancid, sweaty	1858	1415	1373	670	hexanoic acid ^a

1850	floral, rose-like	0	1046	820	1460	geraniol ^a
1886	floral	2946	2415	1801	2027	phenethyl alcohol ^a
1915	floral, violet-like, berry	25	827	870	1003	β -ionone ^a
1942	floral, sweet	870	1058	1912	1042	
1945	rancid, sweaty	195	741	322	514	(Z)-3-hexenoic acid ^a
1977	sweet	12	127	40	23	3-hydroxy-2-methyl-4-pyrone ^c
1980	floral, minty	120	711	422	498	2-phenylethyl 3-methylbutanoate ^a
1987	green, metallic	0	111	165	14	<i>trans</i> -4,5-epoxy-(<i>E</i>)-2-decenal ^d
1995	sweet	1616	1043	1338	1692	γ -nonalactone ^c
2019	strawberry, citrus	1531	1534	1649	1514	
2029	roasted	282	137	345	160	4-hydroxy-2,5-dimethyl-3(2H)-furanone ^d
2034	rancid, sweaty	66	75	43	32	
2049	rancid, sweaty	2733	1527	2140	2621	octanoic acid ^c
2071	phenolic	1304	924	967	1399	
2102	strawberry, sweet	39	58	22	121	
2114	chocolate, roasted	292	794	909	776	
2168	roasted, caramel	3164	2117	3121	3086	2-methoxy-4-vinylphenol ^c
2187	muscat, grape	1340	1117	1038	1299	<i>o</i> -aminoacetophenone ^c
2236	spicy	0	194	979	344	
2250	phenolic	348	88	20	29	
2255	roasted	44	185	215	58	
2272	rancid, sweaty	2514	2645	2207	512	decanoic acid ^c
2330	musty, cucumber	1161	513	281	331	
2370	floral, acid	1336	1267	831	1465	
2380	spicy	0	0	1134	0	
2414	feces	78	171	41	72	indole ^c
2459	feces	1784	1547	1389	1470	3-methylindole ^c
2512	vanilla, chocolate	2176	1891	1710	2298	vanillin ^c
2557	roasted	1210	1144	1142	1293	
2570	roasted	1322	339	1052	905	

2603	acid	1829	699	1224	724
2648	spicy	0	0	1398	0
2839	acid	192	257	166	335
2970	citrus, raspberry	96	1293	1309	1257 4-(4-hydroxyphenyl)-2-butanone ^a

^a Identified by matching RIs and mass spectra and odor qualities with the authentic compounds in DB-WAX and DB-1 column. Tentatively identified by matching; ^b RIs and odor qualities in DB-WAX and DB-1 column, ^c RIs and mass spectra and odor qualities in DB-WAX column, ^d RIs and odor qualities in DB-WAX column, with the authentic compounds.

2-3-1. Hop-Derived Odorants. The GC-O comparison between unhopped and hopped beers revealed 27 components to be hop-derived odorants in beer (**Table 2-2**). Most of these hop-derived substances were common to all three of the beers tested, and some are detected in slight amounts even in unhopped beer. Among them, 15 hop-derived odorants were identified utilizing their Kovats RIs, mass spectra, odor quality agreement with the standard compounds in DB-WAX and DB-1 column, and their absence or rarity in unhopped beer. The mass spectra signals of 4 odorants, 3-methyl-2-butene-1-thiol (MBT), 4MMP, 3MH, and (Z)-1,5-octadien-3-one were too weak to give an unequivocal identification by using the method employed in this study. These odorants were therefore tentatively identified by matching their RIs and odor qualities with those of standard compounds in both DB-WAX and DB-1 column. The remaining 8 odorants could not be identified because they were complex mixtures and/ or were present in insufficient quantities.

Some components are assumed to derive from the metabolism of degraded or isomerized products of hop-derived substances including α -acids, β -acids, polyphenols, and hydrocarbons, during fermentation [53, 54]. Thus, in the current report, one-third of the total amount of hops was added at the beginning of the boiling process, in order to allow any substances generated during wort boiling to persist. Ethyl 2-methylpropanoate, (+/-)-ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 2-phenylethyl 3-methylbutanoate and 4-(4-hydroxyphenyl)-2-butanone were either detected in small amounts or not detected in the wort. The concentrations of these odorants increased after fermentation, and moreover, these components were not detected in the unhopped beer (**Table 2-3**). My results therefore indicate that these compounds were mainly produced by the esterification of hop-derived short-chain acids or by equilibrium reactions with ethanol. Many short-chain acids result from the deterioration of α -acid in hops [104]. This may partly consistent with the contributions of deteriorated hops to the increased hop aroma in beer [60].

Table 2-2. Identified 27 hop-derived potent odorants and threshold values in beer.

RI on DB-WAX	odor qualities detected by GC-O	compounds	difference threshold value (µg/L) ^[10, 94]
1004	citrus, pineapple, sweet	ethyl 2-methylpropanoate	6.3
1068	citrus, apple-like	(+/-)-ethyl 2-methylbutanoate	1.1 ^a
1084	citrus, sweet, apple-like	ethyl 3-methylbutanoate	2.0
1103	green, leafy	1-hexanal	350 ^[65]
1111	almond, roasted	3-methyl-2-butene-1-thiol ^c	0.002 ^[63]
1148	green, leafy	(Z)-3-hexenal	20.0 ^[65]
1180	citrus, pineapple	ethyl 4-methylpentanoate	1.0
1190	resinous	myrcene	9.5
1338	fruity, catty, thiol-like	unknown	
1363	muscat/ blackcurrant-like, fruity	4-mercapto-4-methylpentan-2-one ^c	0.0015
1373	green, metallic	(Z)-1,5-octadien-3-one ^c	0.0034
1383	green	(Z)-3-hexen-1-ol	884
1383	muscat-like	unknown	
1548	floral, citrus, terpenic	(R/S)-linalool	1.0 ^b
1571	green, cucumber	(E,Z)-2,6-nonadienal	0.5 ^[65]
1590	green, metallic	unknown	
1682	fatty	unknown	
1825	fruity, catty, thiol-like	3-mercaptohexan-1-ol ^c	0.055
1850	floral, rose-like	geraniol	4.0
1915	floral, violet-like, berry	β-ionone	0.6
1945	rancid, sweaty	(Z)-3-hexenoic acid	1,300 ^[65]
1980	floral, minty	2-phenylethyl 3-methylbutanoate	88.5
2114	chocolate, roasted	unknown	
2236	spicy	unknown	
2380	spicy	unknown	
2648	spicy	unknown	
2970	citrus, raspberry	4-(4-hydroxyphenyl)-2-butanone	21.2

The values are shown relative to the thresholds in beer [63, 65] and water [11]. ^a The value was determined by using the racemate. ^b The value was determined by using the (R)-isomer. ^c Tentatively identified by matching their RIs and odor qualities with the authentic compounds in DB-WAX and DB-1 column.

Table 2-3. Concentration (µg/L) of quantified hop-derived potent odorants in beer ^a.

compounds	unhopped		Saazer		Hersbrucker		Cascade		CV (%) ^b	detection limit (µg/L) ^c
	wort	beer	wort	beer (aroma unit)	wort	beer (aroma unit)	wort	beer (aroma unit)		
ethyl 2-methylpropanoate	ND ^d	0.27	0.15	3.98 (0.63)	0.45	8.01 (1.27)	0.42	6.39 (1.01)	7.8	0.07
(+/-)-ethyl 2-methylbutanoate	ND ^d	0.02	0.11	1.67 (1.52)	0.16	1.83 (1.66)	0.11	1.20 (1.09)	7.5	0.04
ethyl 3-methylbutanoate	ND ^d	0.01	0.23	5.32 (2.66)	0.23	2.66 (1.33)	0.24	2.13 (1.07)	7.4	0.02
1-hexanal	12.5	6.79	40.2	16.9 (0.05)	33.5	14.2 (0.04)	31.6	14.3 (0.04)	10.3	0.02
(Z)-3-hexen-1-ol	0.01	0.02	7.14	17.6 (0.02)	7.11	18.6 (0.02)	19.1	27.7 (0.03)	3.7	0.01
(R/S)-linalool	ND ^d	ND ^d	27.9	30.3 (15.8<)	71.3	70.5 (36.7<)	52.4	53.9 (28.0<)	4.3	0.05
geraniol	ND ^d	ND ^d	19.8	8.15 (2.04)	14.5	7.37 (1.84)	26.2	12.4 (3.09)	5.7	0.10
β-ionone	ND ^d	ND ^d	0.05	0.16 (0.27)	0.05	0.18 (0.30)	0.06	0.15 (0.25)	2.0	0.005
2-phenylethyl 3-methylbutanoate	ND ^d	0.02	ND ^d	3.05 (0.03)	ND ^d	1.53 (0.02)	ND ^d	2.46 (0.03)	7.1	0.02
4-(4-hydroxyphenyl)-2-butanone	ND ^d	0.11	ND ^d	2.29 (0.11)	ND ^d	1.88 (0.09)	ND ^d	1.37 (0.06)	14.3	0.05

^a The mean value of duplicated analysis. ^b Coefficient of variances were calculated from 12 analysis of same lot beer. ^c Detection limits are the concentration when the height of the signal was three fold of noise. ^d The concentration was under detection limit.

2-3-2. Hop-Derived Odor-Active Components. Among the identified hop-derived odorants, the most intense odor-active components with aroma units greater than 1.0 (**Table 2-3**) and Charm values of more than 1000 (**Table 2-1**) were as follows: linalool, geraniol, ethyl 3-methylbutanoate, (+/-)-ethyl 2-methylbutanoate and ethyl 2-methylpropanoate.

Higher Charm values of greater than 1000 for β -ionone (which had aroma units of greater than 0.3), 4-(4-hydroxyphenyl)-2-butanone, and ethyl 4-methylpentanoate, 3MH, 4MMP, (Z)-1,5-octadien-3-one, (Z)-3-hexenal, and unknown components at RIs of 1383, 1590, 2380 and 2648 were also observed, and taken to be the odor-active components for the hop aroma in beer. In addition, extremely low threshold values were determined for 3MH, 4MMP, and (Z)-1,5-octadien-3-one (**Table 2-2**), which were thus supposed to have effects on the hop aroma, though the author failed to quantify the amount comparable to the threshold value. In support of this contention, Vermeulen [107] detected 3MH and 4MMP in fresh lager, and reported that they had an influence on beer aroma.

2-3-3. Sensory Evaluation. The sensory evaluation examined the intensity of the green, citrus, floral, spicy, and muscat/ blackcurrant-like characteristics, along with the total hop aroma intensity of the beers. **Figure 2-4** shows the characteristics of each cultivar as an average intensity of the individual panelists' scores, and the detailed data for the **Figure 2-4** are shown in **Table 2-4**.

The results show that citrus and floral notes characterized the hop aroma of Saazer beer. Hersbrucker beer was characterized by spicy, green and floral notes, and the score of spicy characteristic was significantly higher than other two cultivars with the t-test value below 0.003, while that of citrus characteristics was lower.

Cascade beer was characterized by muscat/ blackcurrant-like and citrus notes. The significantly higher intensity of the muscat/ blackcurrant-like characteristic than other two cultivars was observed with the t-test value below 0.001. The sensory score for total intensity of aroma was highest for Cascade with the t-test value below 0.02, followed by Saazer, and then Hersbrucker.

Contributor to each of these characteristics is discussed in detail, along with the associated Charm value data, in the following sections.

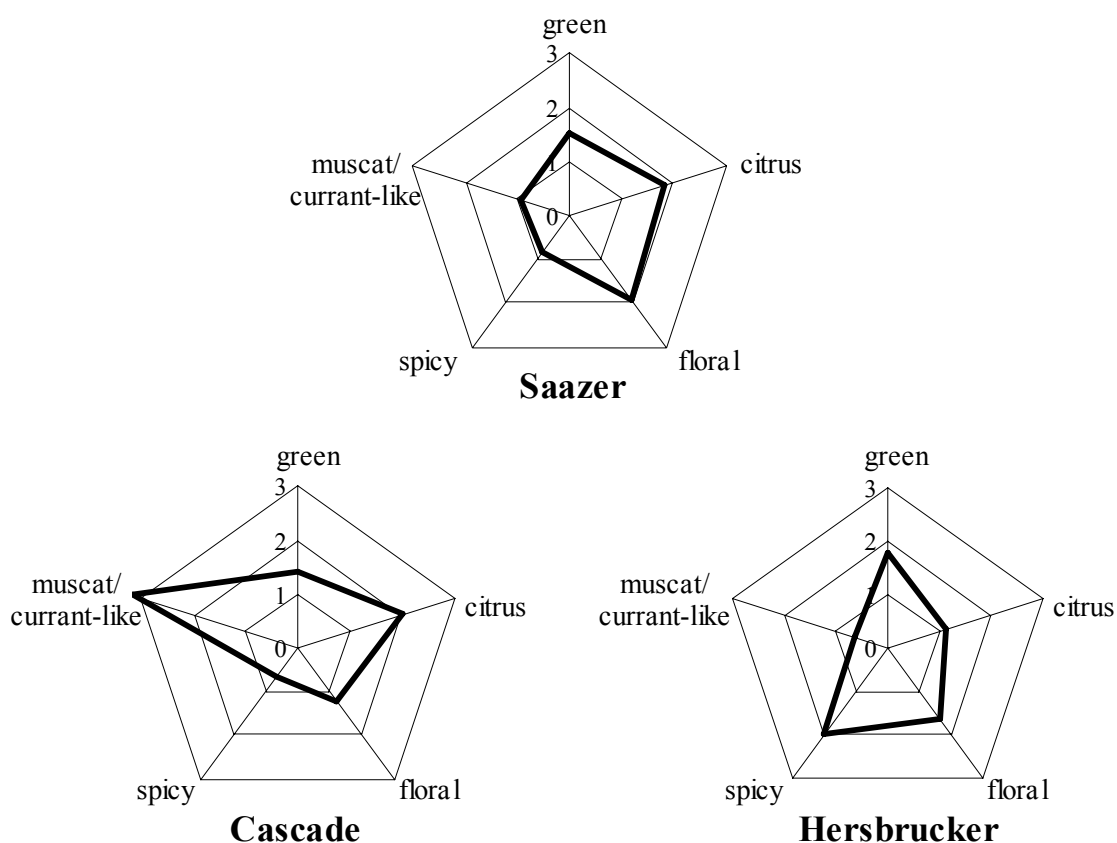


Figure 2-4. Aroma-profile of beers hopped with Saazer, Cascade, and Hersbrucker cultivar.

Table 2-4. Intensity and t-test value for the characteristics of beers brewed with different hop cultivars.

	intensity ^a			t-test value ^b		
	Saazer	Hersbrucker	Cascade	Saazer : Hersbrucker	Hersbrucker : Cascade	Cascade : Saazer
green	1.50 ± 0.52	1.79 ± 0.80	1.43 ± 0.71	0.41	0.38	0.85
citrus	1.79 ± 0.75	1.14 ± 0.41	2.00 ± 0.71	0.08	0.04	0.45
floral	1.93 ± 0.49	1.64 ± 0.66	1.21 ± 0.42	0.32	0.25	0.02
spicy	0.86 ± 0.49	2.00 ± 0.92	0.64 ± 0.26	0.003	0.002	0.08
muscat/ blackcurrant-like	0.93 ± 0.38	0.64 ± 0.52	3.14 ± 0.68	0.23	0.00001	0.0002
total hop aroma intensity	2.07 ± 0.45	1.79 ± 0.76	3.07 ± 0.66	0.28	0.01	0.02

^a Mean intensity value of the scores from 7 panelists ± standard deviation of the mean value.

^b Paired t-test value comparing Saazer and Hersbrucker beer, Hersbrucker and Cascade beer, Cascade and Saazer beer.

2-3-4. Green Characteristic. Green odorants were observed for 1-hexanal, (*Z*)-3-hexenal, (*E,Z*)-2,6-nonadienal, (*Z*)-3-hexen-1-ol, and (*Z*)-1,5-octadien-3-one, and unknown odorant at RI 1590 by the GC-O analysis. The aldehydes and alcohol listed above have been described as odorants present in green leaves [40]. In addition, (*Z*)-3-hexenal and (*Z*)-1,5-octadien-3-one [89], and 1-hexanal [42] have previously been reported in hops. The (*Z*)-3-hexen-1-ol content in beer increased after fermentation, and so is assumed to be generated from compounds such as (*Z*)-3-hexenal [40]. The total of the Charm values derived from green odorants present in Hersbrucker beer was greater than that for Saazer and Cascade, which was consistent with the results of the sensory evaluation. The concentrations of (*Z*)-3-hexen-1-ol, 1-hexanal themselves were lower than the threshold values as shown in **Table 2-3**, however, the relation between the result of total Charm value and sensory evaluation indicates that the sum of these odorants comprise the green aroma of over the threshold [9].

2-3-5. Muscat/ Blackcurrant-Like Characteristic. Cascade beer was identified to have a muscat/ blackcurrant-like characteristic according to the results of the sensory analysis. Intense muscat/ blackcurrant-like odorants, which had Charm values of more than 1000, were detected at RIs of 1363 and 1383 by GC-O. The former odorant was tentatively identified as 4MMP, which was revealed to have an extremely low threshold value in beer (**Table 2-2**), and recently 4MMP was detected in Cascade hops [91]. It was assumed to have an effect on the aroma as it does in wine [18], though the author was unable to quantify the threshold amounts of the compound.

The second muscat-like flavor was identified at RI 1383, where (*Z*)-3-hexen-1-ol was identified by GC-MS. (*Z*)-3-hexen-1-ol itself was confirmed as the odor-active and green odorant by the Charm analysis employing a DB-1 column. It has also been described as one of the major volatiles in muscat grape flavor [50], and the higher concentration was observed in Cascade beer. Thus, (*Z*)-3-hexen-1-ol in combination with the unknown odorant at RI 1383 was assumed to be a contributor to the muscat flavor. The sum of the Charm values of these two RIs in the Cascade-hopped beer was higher than that of the other two beers tested: this indicated that these components were the main contributors to the muscat/ blackcurrant-like characteristic. Geraniol, which itself has a floral characteristic, has also been described as a major volatile in muscat grape flavor [11, 50]. As the author found it at a higher Charm value and concentration, it was assumed to be a contributor to this characteristic odor.

2-3-6. Spicy Characteristic. As shown in **Figure 2-4**, Hersbrucker was also strongly characterized by a spicy aroma. Spicy odorants were detected by CharmAnalysisTM at RIs of 2236, 2380 and 2648, which is a region where sesquiterpenoids are abundant. My observations were consistent with a previous report in which the sesquiterpenoid fraction of Hersbrucker was thought to yield a spicy characteristic [20, 36]. Components at RIs of 2380 and 2648 were detected only in Hersbrucker. The sum of the Charm values of the spicy characteristics of Hersbrucker was significantly higher than those of the other two types of beer. Thus, the author demonstrate that these components contribute to the spicy characteristic, although they could not be identified in the current study.

2-3-7. Floral Characteristic. Linalool, geraniol, and β -ionone were shown to contribute to the floral note, based on the Charm values and the aroma units. In the current study, the threshold value of linalool, 1.0 $\mu\text{g/L}$, was determined using the (*R*)-isomer. Though enantiomeric quantification of the beer was not performed here, the reported enantiomeric ratio of the (*R*)-isomer in beer is greater than 52 % [90]. As for Saazer beer, which was observed to have the lowest concentration of linalool, 30.3 $\mu\text{g/L}$, among the three cultivars, the calculated concentration of the (*R*)-isomer and aroma unit of linalool was greater than 15.8 $\mu\text{g/L}$ and 15.8 respectively (**Table 2-3**).

In addition to these three terpenoids, 2-phenylethyl 3-methylbutanoate was also associated with the floral characteristic. The component was not detected in wort, and so is assumed to be generated during fermentation from compounds such as 3-methylbutanoic acid and 2-phenylethanol [11]. The sensory score for floral attributes was highest for Saazer, followed by Hersbrucker, and then Cascade, thus was not consistent with the total Charm values of these components. This indicated that additional components from hops and other raw materials might contribute synergistically or antagonistically to the floral characteristic. Further investigations will be required to clarify this issue.

2-3-8. Citrus Characteristic. Ethyl 3-methylbutanoate, ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, 4-(4-hydroxyphenyl)-2-butanone, ethyl 4-methylpentanoate, linalool, 3MH (which was revealed to have an extremely low threshold value), and an unknown component at RI 1338 were identified as odorants contributing to the citrus flavor. The remarkably higher Charm values of ethyl 3-methylbutanoate and ethyl 4-methylpentanoate (**Table 2-1**), and aroma unit of ethyl 3-methylbutanoate (**Table 2-3**) were observed in Saazer beer than in the other two cultivars. The citrus score for Cascade according to the organoleptic estimation was higher than that for the other

beers, which could not be explained by the sum of the Charm values. Additional components, both from hops and other raw materials, are therefore likely to contribute to this characteristic, either synergistically or antagonistically.

In order to reveal the contributions of these components in more detail, identification of the unknown components mentioned above, and quantification of the components with extremely low threshold value, and investigation from enantiomeric viewpoint, followed by aroma simulations recombining the odorants will be required. This might also allow the characterization of novel odorants.

Chapter 3

Comparison of 4-Mercapto-4-methylpentan-2-one Contents in Hop Cultivars from Different Growing Regions

3-1. INTRODUCTION

To determine the components which contribute to the sensory hop aroma characteristics in beer, hop-derived potent odorants that persist even after fermentation and comprise hop aroma characteristics of beer were examined in the Chapter 2. The blackcurrant/ muscat-like aroma, and floral aroma were predominant in sensory evaluation of strongly-hopped Cascade beer, and geraniol and a thiol, 4MMP were identified as contributors to its character.

Thiols are well known for their extremely low threshold values (below 100 ng/L), and contributions to the fruity aroma of beer or wine [18, 92, 93, 99, 102, 107]. In hops, the occurrence of 4MMP and its content was reported only for Cascade cultivar before [93], however, had not been investigated for other cultivars. Furthermore, the reason why 4MMP exists at higher content in Cascade cultivar had not been studied in previous studies. In this chapter, the author examined the 4MMP content in hops depending on cultivars and their growing regions in order to establish a distinctive hop aroma characteristic in beer.

3-2. MATERIALS AND METHODS

3-2-1. Reagents. (+/-)-ethyl 2-methylbutanoate (99.0 %) and *p*-hydroxymercuribenzoic acid sodium salt (98.0 %) were purchased from Acros Organics (Morris Plains, NJ). Dowex® (1 × 2, Cl(-)-form, strongly basic, 50–100 mesh) was obtained from Sigma-Aldrich (St Louis, MO). 4MMP (stored at 1% (w/w) in triacetin), (*S*)-(+)-linalool (87.0 %), and 4-methoxy-2-methyl-2-mercaptobutane (99.0 %) were obtained from San-Ei Gen FFI, Inc. (Osaka, Japan). *tert*-butyl-4-methoxyphenol (98.0 %) and L-cysteine hydrochloride monohydrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (*R/S*)-linalool (95.0 %), (*R*)-(-)-linalool (98.5 %), geraniol (99.0 %), β-damascone (90.0 %), ethyl 4-methylpentanoate (97.0 %), and (*Z*)-3-hepten-1-ol (95.0 %) were

purchased from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane (99.0 %), HNO₃ for inductively-coupled plasma mass spectroscopy (ICP-MS) analysis, yttrium, and indium for atomic-absorption analysis, granular copper (99.9 %) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). (S)-(+)-ethyl-2-methylbutanoate (85.0 %) was obtained from T. Hasegawa Co., Ltd. (Tokyo, Japan). 3-mercaptohexan-1-ol (97.0 %) was purchased from Avocado Research Chemicals Ltd. (Heysham, UK). 3-mercaptohexyl acetate (95.0 %) was purchased from Atlantic Research Chemicals Ltd. (Bude, UK).

3-2-2. Hops. The hop cultivars Simcoe (2005 and 2006 crops, USA) and Topaz (2007 crop, Australia) were purchased from Yakima Chief (Sunnyside, WA) and Hop Products Australia (Tasmania, Australia), respectively. Hersbrucker (2005 crop, Germany), Saazer (2005 crop, Czech Republic), Fuggle (2005 crop, UK), and Perle (2006 crop, Germany) were purchased from Joh. Barth & Sohn GmbH & Co. (Nuremberg, Germany). Summit (2006 crop, USA), Millennium (2006 crop, USA), and Nugget (2005 crop, USA) were purchased from John I. Haas, Inc. (Yakima, WA). Apollo (2006 crop, USA), Cascade (2006 crops, USA), Willamette (2006 crop, USA), and Perle (2006 crop, USA) were purchased from S. S. Steiner, Inc. (New York, NY). Magnum (2006 crop, Germany), Taurus (2006 crop, Germany), Nugget (2006 crop, Germany), and Pacific Gem (2006 crop, New Zealand) were purchased from Simon H. Steiner, Hopfen, GmbH (Mainburg, Germany).

3-2-3. Brewing Processes. Pellets of the hop cultivars Simcoe (2005 crop), Summit, Apollo, Millennium, Cascade, Willamette, Magnum, Taurus, Hersbrucker, Saazer, and Fuggle were used in the brewing process. Unhopped beer and beers hopped with each cultivar were brewed independently. For the evaluation of cultivars, a 20-L volume of wort without hops was boiled for 60 min in a wort kettle; a 40-g sample of hops was then added after the wort-cooling process, in order to examine the properties of the hop components and the aroma characteristics independent of any temperature effect. Static fermentations were carried out in 5 L stainless-steel tall tubes as described in the Chapter 2.

3-2-4. Sensory Evaluation. Sensory evaluation of the hopped beers was performed by nine trained panelists, using the intensity of unhopped beer as a control. The panelists were asked to describe the hop aroma characteristics, and to evaluate the intensities of the ‘blackcurrant-like’ aroma and the total hop aroma. The responses to each description were shown to be consistent among the panelists using a matching test [66]. The odor intensities were rated on the following scale (with 1.0-interval steps): 0 = not perceivable; 2 = weak; 4 = normal; 6 = strong; 8 = very strong. The cultivar characteristics were compared using the mean intensity values of the scores.

3-2-5. Solvent-Assisted Flavor Evaporation (SAFE). SAFE apparatus (**Figure 3-1a**; Kiriya Glass Works Co., Tokyo, Japan) designed to the specifications reported by Engel *et al.* [29] was used to prepare extracts for GC-O analysis. The volatiles were isolated from 150 ml samples of unhopped, Magnum, Summit, Simcoe, and Apollo beers. The SAFE apparatus was connected to a 500-ml distillation flask and a 500-ml receiving flask (**Figure 3-1b**). The distillation flask was warmed to 35 °C by pumping water from a heated reservoir through the jacketed body of the apparatus. The receiving vessel and cold trap were cooled by liquid nitrogen, and the apparatus was evacuated under a high vacuum (1.5×10^{-5} torr) by a VPC-250F diffusion pump (Ulvac Kiko, Inc., Yokohama, Japan). Each sample was added dropwise via the sample reservoir into the distillation flask to prevent excessive foaming. After distillation, the vacuum was released, and the surface of the cold trap was washed with 10 ml ethanol. The distillate was then applied to isolate thiols using resin and sodium *p*-hydroxymercuribenzoate (*p*HMB) as described below.

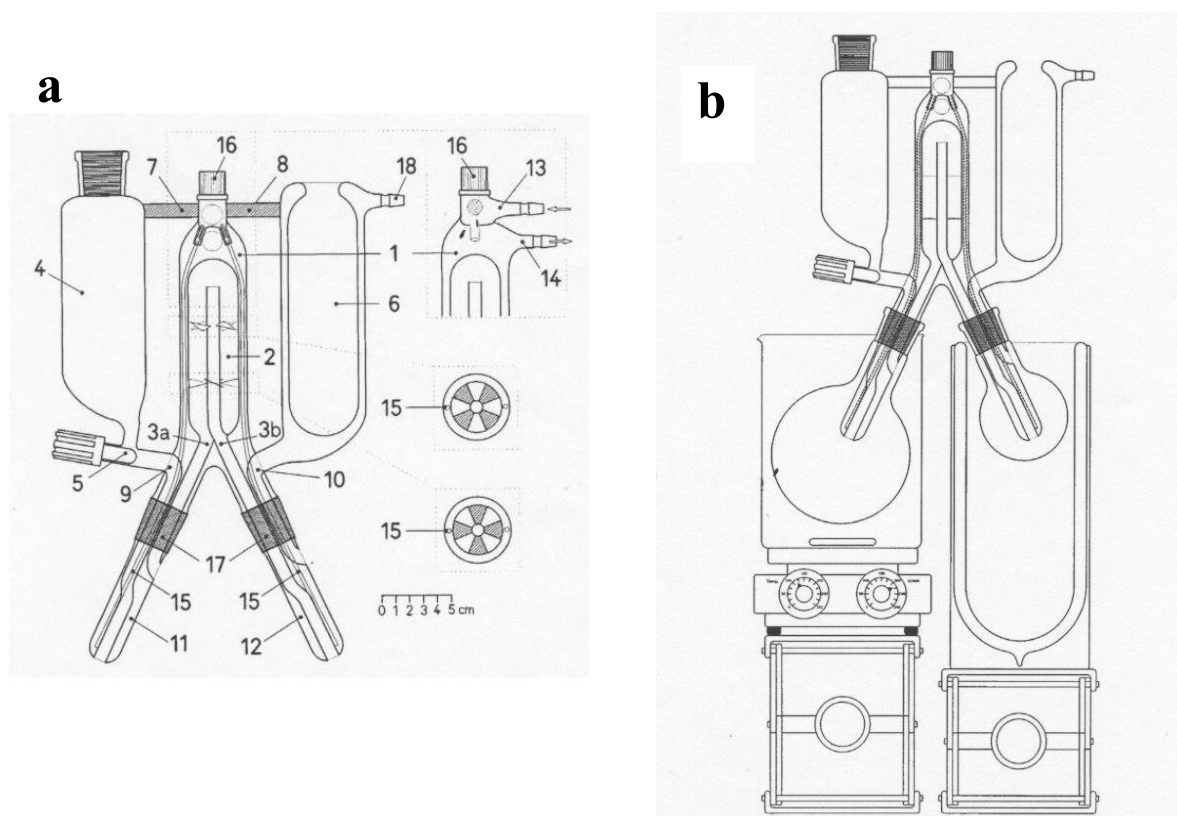


Figure 3-1. (a) Schematic view of solvent-assisted flavor evaporation (SAFE) [29]. The apparatus (size, 40 x 25 x 7 cm) consists of ; No.4, dropping funnel; No. 6, cooling trap; No. 2, central head; Nos. 11 and 12, bearing two legs; No. 17, ground joints NS 29 to fix distillation and receiving vessels. The outlet of the dropping funnel leads to the bottom of the No. 11 left leg. The vapor inlets to the head (No. 3a) and the inlet to the trap are mounted on the sides of each leg. To ensure a constant temperature during distillation

and to prevent condensation of the volatiles, the head (No. 2) and the two legs (No. 11 and 12) are completely thermostated with warm water. From the water inlet (No. 13), two flexible polyethylene tubes (No. 15) guide the water flow to the bottom of both legs (No. 11 and 12) to afford effective temperature regulation by avoiding the formation of air bubbles [29]. (b) View of the assembled equipment for SAFE with water bath, distillation flask and receiving flask [29].

3-2-6. Isolation of Volatile Thiols. The volatile thiols were isolated using a strongly basic anion-exchanger resin (Dowex 1) with elution columns (Kiryama Glass Works Co.), which were designed according to the description given by Tominaga *et al.* (**Figure 3-2**) [101].

For the GC-O analysis, 0.02 mM *tert*-butyl-4-methoxyphenol was added as an antioxidant to 150 ml SAFE distillate, which was employed for the subsequent extraction with *p*HMB. For the quantifications of 4MMP in beer by GC-MS, 100 ml beer samples containing 1.0 µg/L 4-methoxy-2-methyl-2-mercaptobutane as an internal standard and 0.02 mM *tert*-butyl-4-methoxyphenol were subjected to degassing by sonicating for 20 min, and then were applied for the following extraction with *p*HMB. For the quantifications of 4MMP in hop pellets by GC-MS, aroma components were extracted from 2 g hop pellets with 100 ml 40 °C water for 30 min. The water extracts with 0.02 mM *tert*-butyl-4-methoxyphenol were applied for the following extraction with *p*HMB. The extractions using the *p*HMB solution and Dowex 1 columns were performed by the method described previously by Tominaga *et al.* [101].

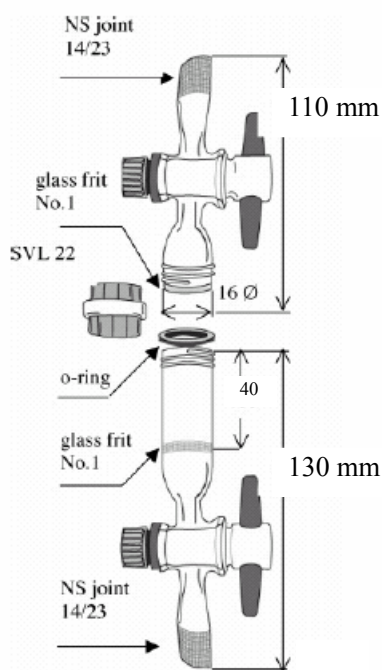


Figure 3-2. The elution column designed for the isolation of volatile thiols [101].

3-2-7. GC-O Analysis. GC-O analysis of the extracts prepared using SAFE apparatus, Dowex 1, and *p*HMB from unhopped, Magnum, Summit, Simcoe, and Apollo beers was performed by CharmAnalysisTM (Datu Inc., Geneva, NY), as described in the Chapter 2.

Identification of the hop-derived thiols was attempted by comparing their odor qualities, RIs, and mass spectra with those of authentic compounds on both DB-WAX and HP-5 capillary columns (Agilent Technologies, Santa Clara, CA; length = 30 m; i.d. = 0.25 mm; film thickness = 0.25 μ m) by GC-MS (Agilent 6890 gas chromatograph coupled to an Agilent MSD5973N quadrupole mass spectrometer).

3-2-8. Quantification of Thiols by Multidimensional (MD)-GC-MS. 4MMP quantification was performed by MD-GC-MS using an Agilent 6890 gas chromatograph (Agilent Technologies) equipped with a first column, a multicolumn switching system (MCS2; Gerstel, Mulheim a/d Ruhr, Germany), and an Agilent 6890 GC coupled to a MSD5973N quadrupole mass spectrometer equipped with a second column.

A 1- μ L sample was injected and extract separation was performed on the first column (DB-5MS capillary column; 30 m length \times 0.25 mm i.d.; film thickness = 0.25 μ m; Agilent Technologies). The inlet temperature was set at 250 $^{\circ}$ C with splitless injection. The oven temperature was programmed to rise from 40 $^{\circ}$ C (held for 1 min) to 160 $^{\circ}$ C (held for 16 min) at a rate of 10 $^{\circ}$ C/min, and then to 300 $^{\circ}$ C (held for 10 min) at a rate of 10 $^{\circ}$ C/min with a constant carrier helium gas flow (1 ml/min).

At the elution of 4MMP, the effluent was transferred to a cold trap at -100 $^{\circ}$ C using the MCS2 cooled by liquid nitrogen. After cooling, the trapped material was further separated by the second column (DB-WAX capillary column; 30 m length \times 0.25 mm i.d.; film thickness = 0.25 μ m; Agilent Technologies). The oven temperature was programmed to rise from 40 $^{\circ}$ C (held for 15 min) to 160 $^{\circ}$ C (held for 16 min) at a rate of 5 $^{\circ}$ C/min, and then to 230 $^{\circ}$ C (held for 7 min) at a rate of 10 $^{\circ}$ C/min with a constant carrier helium gas flow (1 ml/min). The GC-MS system was operated in the electron-impact mode at 70 eV, with the SIM mode at m/z 132 for 4MMP, and at m/z 100 for 4-methoxy-2-methyl-2-mercaptobutane.



Figure 3-3. MD-GC-MS system using an Agilent 6890 gas chromatograph equipped with a first column, a multicolumn switching system, and an Agilent 6890 GC coupled to a MSD5973N quadrupole mass spectrometer equipped with a second column.

3-2-9. Quantification of Esters and Terpenoids. The quantification of myrcene, (*R/S*)-linalool, and geraniol in the beer samples was carried out by the SBSE method using β -damascone as an internal standard, as described in the Chapter 1. The amount of (+/-)-ethyl 2-methylbutanoate in the beer samples was measured by the liquid-extraction method with dichloromethane using (*Z*)-3-hepten-1-ol as an internal standard, as described in the Chapter 1. The data are shown as the mean values of duplicate analyses.

3-2-10. Quantification of Ethyl 4-methylpentanoate. Ethyl 4-methylpentanoate was quantified using the large-volume dynamic-headspace method with GC-MS and the Entech 7100A system (Entech, Simi Valley, CA). Methyl propionate was added to each beer sample at a final concentration of 1.25 mg/L as an internal standard. Then, 2 ml of the beer sample including the internal standard was diluted with 98 ml distilled water. The 100-ml diluted sample was then transferred into a 250-ml jar containing a glass bubbling tube. Helium gas (750 ml) was bubbled into the jar at a temperature of 40 °C and a flow rate of 60 ml/min. A three-stage concentration method was used to remove excess water and carbon dioxide from the stream of volatiles from the jar, which was subsequently introduced into the preconcentration system. The flow was initially concentrated in a cryogenic trap consisting of glass beads and Tenax (Module 1) at 20 °C. The trap was then heated to 180 °C, and the concentrated volatiles were transferred by passing helium gas into a secondary Tenax trap (Module 2) that was held at 20 °C. The trap was then heated to 180 °C for 3.5 min, and the concentrated

volatiles were transferred into an inert empty glass tube (Module 3) that was held at $-150\text{ }^{\circ}\text{C}$. Separation of the volatiles was performed with an Agilent 6890 gas chromatograph coupled to a MSD5973N quadrupole mass spectrometer (Agilent Technologies) equipped with a DB-1 capillary column (60 m length \times 0.32 mm i.d.; film thickness = 1.0 μm ; Agilent Technologies) with a helium carrier gas (1.2 ml/min). The third trap was heated to $150\text{ }^{\circ}\text{C}$ for 4 min in order to inject the volatiles into the GC-MS apparatus. The volatiles were injected using a pulsed split mode with a split ratio of 15:1. The oven temperature was programmed to rise from $40\text{ }^{\circ}\text{C}$ (held for 5 min) to $300\text{ }^{\circ}\text{C}$ (held for 5 min) at a rate of $10\text{ }^{\circ}\text{C}/\text{min}$. The GC-MS system was operated in the electron-impact mode at 70 eV, with the SIM mode at m/z 88.

3-2-11. Determination of Enantiomeric Excess (ee) Values. The ee values of linalool and ethyl 2-methylbutanoate were investigated using MD-GC-MS as described above, employing a DB-WAX capillary column (60 m length \times 0.32 mm i.d.; film thickness = 0.25 μm ; Agilent Technologies) as the first column, and an RT-BetaDEXse chiral column (30 m length \times 0.32 mm i.d.; film thickness = 0.25 μm ; Restek, Bellefonte, PA) as the second column. The inlet temperature was set at $250\text{ }^{\circ}\text{C}$ with splitless injection, and 1 μL samples were injected.

The oven temperature of the first column was programmed to rise from $40\text{ }^{\circ}\text{C}$ (held for 2 min) to $220\text{ }^{\circ}\text{C}$ at a rate of $3\text{ }^{\circ}\text{C}/\text{min}$, with a constant carrier helium gas flow (1 ml/min). On the second (chiral) columns, separation of ethyl 2-methylbutanoate and linalool was performed by isothermally maintaining temperatures of $55\text{ }^{\circ}\text{C}$ and $90\text{ }^{\circ}\text{C}$, respectively. The GC-MS system was operated in the SIM mode at m/z 102 for ethyl 2-methylbutanoate and at m/z 93 for linalool. The ee value was calculated based on the integrated peak area of the *R*-isomer and the *S*-isomer using the following formula: $\text{ee (\%)} = ([R] - [S]) / ([R] + [S]) \times 100$.

3-2-12. Quantification of Divalent Metal Ions. Divalent metal ions were quantified using ICP-MS (Agilent 7500c). Prior to analysis, samples were digested in closed vessels made of polytetrafluoroethylene (PTFE) using the Multiwave 3000 microwave sample digestion system (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA).

The PTFE vessels were washed with 6 ml HNO_3 and subjected to microwave digestion programmed at 1250 W (held for 10 min) and then 0 W (held for 20 min). The vessels were then rinsed with ultrapure water and filled with 1 N HNO_3 . The polypropylene tubes were washed by filling with alkaline detergent overnight, followed by 1 N HNO_3 overnight, and then rinsing with ultrapure water.

Samples of hop pellets (200 mg) with 5 ml HNO_3 and internal standards (yttrium or indium) were

hermetically sealed in the PTFE vessels, and subjected to microwave digestion (Multiwave 3000). The microwave was programmed at 0 W (held for 10 min), 1400 W (held for 40 min), and then 0 W (held for 20 min). The PTFE vessels were left to cool, and the digested samples were transferred to polypropylene tubes by washing with ultrapure water and adjusting the volume to 20 ml. The samples were then filtered with cellulose nitrate 0.2 μm filters (Advantec, Tokyo, Japan).

The samples were subjected to ICP-MS analysis with an Agilent 7500c under the indicated plasma conditions (radio frequency (RF) output = 1500 W; RF matching = 1.75 V; carrier gas flow = 0.8 L/min; makeup gas flow = 0.2 L/min; microflow as nebulizer at 100 $\mu\text{L}/\text{min}$; scotchamber temperature = 2 $^{\circ}\text{C}$), ion-lens conditions (pull-out voltage = 3.4 V; einzel1.3 = -100 V; einzel2 = 15 V; incidence into the cell = -22 V; output from the cell = -15 V; plate bias = -45 V), octapole conditions (RF = 200 V with a bias of -8 V), quadrupole parameters (atomic mass unit (AMU) gain = 129; AMU offset = 124; quadrupole bias = -7 V), and detector conditions (-8 mV discriminator, 1670 V analog high voltage (HV), 1100 V pulse HV). Helium (2 ml/min) was used as the reaction gas for copper and zinc, 3 ml/min helium was used for iron, and 2 ml/min hydrogen was used for manganese.

3-2-13. Lead-Conductance Value of Hop Pellets. The lead conductance values of the hops were determined using the EBC 7.4 method [30].

3-3. RESULTS AND DISCUSSION

The 17 hop cultivars examined in this chapter, their origins, crop year and abbreviations are listed in **Table 3-1**. Of the 17 cultivars, 11 were evaluated in beer as well as the analysis of hop pellets.

Table 3-1. Abbreviations, origins, crop years, and lead conduct values of α -acids (%) of hops cultivars.

Cultivar	Country	Abbreviation	Crop year	Lead conduct value of α -acids (%)
Simcoe	USA	US- SIM	2005	10.4
			2006	10.4
Summit	USA	US- SUM	2006	16.3
Apollo	USA	US- APO	2006	17.1
Millenium	USA	US- MIL	2006	15.1
Cascade	USA	US- CAS	2006	5.5
Willamette	USA	US- WIL	2006	3.4
Topaz	Australia	AU- TPZ	2007	13.6
Pacific Gem	New Zealand	NZ- PGM	2006	14.0
Perle	USA	US- PEL	2006	7.0
Perle	Germany	GE- PEL	2006	10.7
Nugget	USA	US- NUG	2005	11.9
Nugget	Germany	GE- NUG	2006	11.3
Magnum	Germany	GE- MAG	2006	12.5
Taurus	Germany	GE- TAU	2006	13.7
Hersbrucker	Germany	GE- HER	2005	2.9
Saazer	Czech Republic	CZ- SAZ	2005	5.4
Fuggle	UK	UK- FGL	2005	4.6

3-3-1. Blackcurrant-Like Aroma of Beers. The intensities of blackcurrant-like aroma and total hop aromas were evaluated in beers hopped with the 11 hop cultivars, as shown in **Figure 3-4a** and **3-4b**. Hops were added after the wort cooling process, in order to investigate the hop-derived aroma qualities which remain even after fermentation, and obtain objective analytical data without the effect of temperature.

The fruity blackcurrant-like aroma was highest in the beer hopped with USA cultivars, Summit and Simcoe, followed by Apollo and Cascade. In addition, a pungent green onion-like, sulfur-like aroma was also detected only in Summit and Apollo beers (data not shown). ‘Blackcurrant-like’ characteristic is often attributed to the presence of thiols in beer and wine [99, 100, 106, 107]. The blackcurrant-like characteristics, as well as the pungent green onion-like aroma, extremely decreased in perception, when copper granulars was added to the beers, suggesting that thiols were possible contributors to these characteristics [107]. Therefore the author examined blackcurrant-like thiols in beers, and contributors to the characteristics of Simcoe, Summit, Apollo by GC-O analysis.

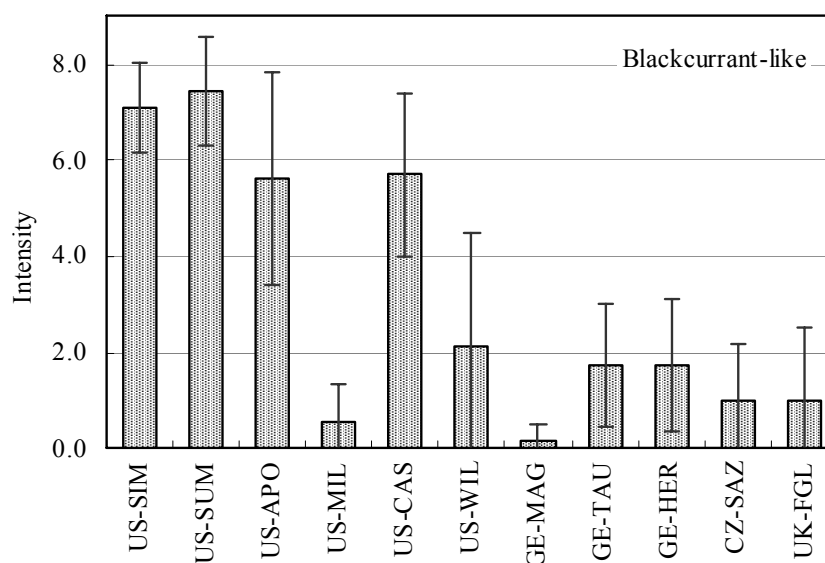


Figure 3-4a. Intensities of the blackcurrant-like aroma evaluated in sensory analysis.

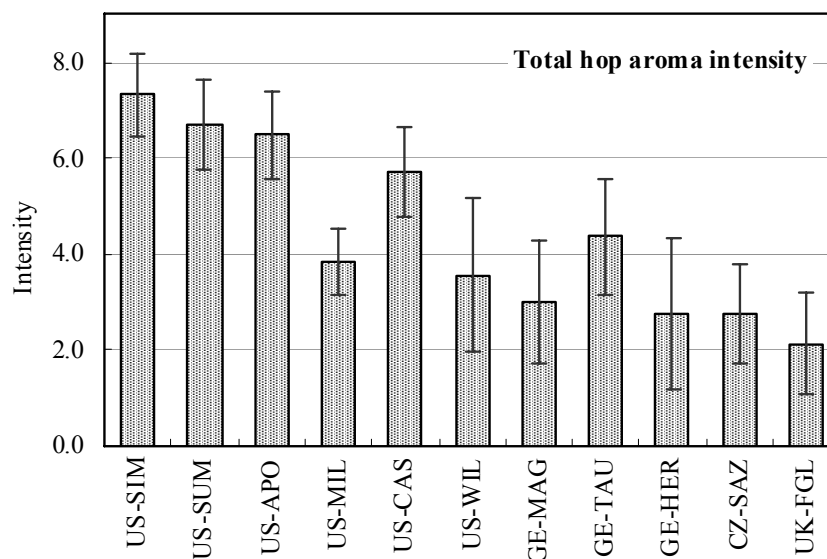


Figure 3-4b. Intensities of the total hop aroma evaluated in sensory analysis.

3-3-2. GC-O Analysis of Beers. The extracts from beers hopped with Summit, Apollo, Simcoe, Magnum and unhopped beer were prepared by the method which extract thiols specifically. In the preparation of the beer extracts for GC-O analysis, the SAFE apparatus was used to prevent the formation of artifacts from nonvolatile compounds in the GC-O inlets, then the extraction was performed using strongly basic anion exchanger resin (Dowex 1) and *p*HMB.

CharmAnalysisTM was used in GC-O analysis since it allows aroma components to be carried on air flowing at 30 ml/min [5], the flow of the odorants does not stay at the sniff port, and the boundaries between the aroma components are clearly defined. Chromatographic peaks for each extract are generated in CharmAnalysisTM, and the peak areas were integrated to yield the Charm values (**Table 3-2**) [3].

The blackcurrant-like odorants in beer extracts detected by GC-O analysis are shown in **Table 3-2**. Of the 7 components listed, four odorants (RIs at 1208, 1442, 1515, 1719) were novel to the current study, and the other three were described in the Chapter 2. The components at RIs of 1377, 1719, 1840 was identified as 4MMP (**Figure 3-5a**), 3-mercaptohexyl acetate (3MHA: **Figure 3-5c**), 3MH (**Figure 3-5b**) respectively by comparing odor qualities, RIs and mass spectra with those of authentic compounds on both DB-WAX and HP-5 capillary columns by GC-MS.

With the exception of 3MHA, the remaining 6 odorants were detected with little or no Charm value in unhopped beer, but were increased by the addition of hops, indicating that they were hop-derived components. Of the 6 blackcurrant-like odorants derived from hops, 3 components

including 4MMP were not detected in the extract from Magnum where blackcurrant-like aroma was not detected in sensory evaluation. Furthermore, extremely higher Charm value was detected at 4MMP, and therefore 4MMP was supposed as the main contributor to the character.

Table 3-2. Charm values of blackcurrant-like odorants extracted from beer.

RIs		Characters detected by GC-O	Charm Values					Identified compounds
DB -WAX	HP-5		US- SIM	US- APO	US- SUM	GE- MAG	Un- hopped	
1208		blackcurrant-like, passion fruit-like	414	360	444	0	0	
1342		blackcurrant-like, passion fruit-like	400	306	452	303	0	
1377	933	fruity, blackcurrant-like	990	765	777	65	0	4-mercapto-4-methyl -pentan-2-one
1442		blackcurrant-like, passion fruit-like	143	180	162	0	0	
1515		blackcurrant-like	123	131	144	177	48	
1719	1249	blackcurrant-like, grapefruit-like	371	411	401	387	456	3-mercaptohexyl acetate
1840	1121	blackcurrant-like, grapefruit-like	364	406	389	316	153	3-mercaptohexan-1-ol

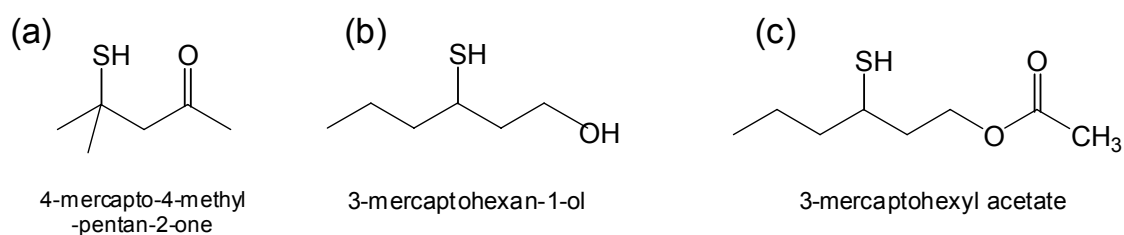


Figure 3-5. The structures of 4-mercapto-4-methylpentan-2-one, 3-mercaptohexan-1-ol, 3-mercaptohexyl acetate.

3-3-3. 4MMP Contents of Beers. The concentrations of 4MMP in beers hopped with 11 cultivars (**Table 3-3**) were determined by the method using Dowex 1, *p*HMB and MD-GC-MS. In beers, as shown in **Table 3-3**, extremely high contents of 4MMP were observed in beers hopped with Simcoe (183.8 ng/L), followed by Summit (116.4 ng/L), Apollo (109.2 ng/L) and Cascade (16.9 ng/L), where the ‘blackcurrant-like’ characteristic was strong in the sensory evaluation (**Figure 3-4**).

In wine, 4MMP is believed to exist as cysteine conjugate [97], and released from the precursor during fermentation processes. The changes in 4MMP concentrations during fermentation were observed for Simcoe beer as depicted in **Figure 3-6**. The 4MMP content increased by 33% during fermentation, and this indicates that most 4MMP exist as freely in wort or in hop pellets and only a small amount is formed during fermentation process from precursors. The 4MMP content peaked during the early stages of fermentation (**Figure 3-6**), as was also observed in wine [95]. Cysteine conjugate precursors present in hops or wort are thought to be transported into yeast cells along with other amino acids, and then released by enzymatic activity, as suggested by a previous report on wine aroma [96], considering the distinguishability from other amino acids, optimal pH and concentration of the enzyme activity in wort.

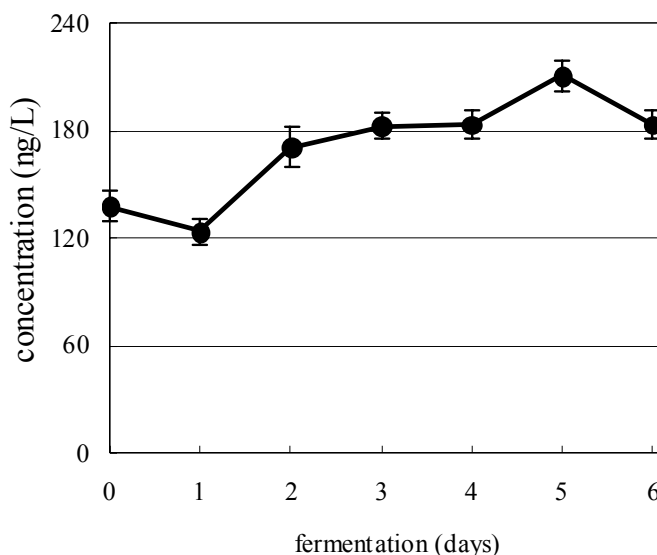


Figure 3-6. Changes in 4-mercapto-4-methylpentan-2-one concentrations during fermentation of US-SIM wort.

In this chapter, the author investigated the contributions of 4MMP to both the blackcurrant characteristic and the total hop aroma intensity. Based on the extremely low threshold value in beer (1.5 ng/L) determined in the Chapter 2, 4MMP was expected to be the main contributor to the blackcurrant-like characteristic. The 4MMP contents of the beers also appeared to affect the overall hop aroma intensity, as shown in **Figure 3-4b**; the highest intensity was observed in Simcoe, followed by Summit, Apollo, and then Cascade. The author investigated the components previously reported to contribute to hop aroma, as shown in **Table 3-3**, and found no associations with total hop aroma intensity. For instance, myrcene, which is reportedly one of the most odor-active components in hop cones [89] with a threshold value in beer of 9.5 µg/L [Chapter 5], was present at the highest levels in Taurus and Summit beers. Linalool, which is reportedly a key odorant in hops [89, 90] that is also found in beers [90], was present at the highest level in Taurus beer followed by Simcoe beer; its ee value (%) [90], which influence the threshold value and therefore the aroma impact, did not differ significantly between cultivars (**Table 3-3**). Geraniol, which is a major contributor to hop aroma [60], was found at the highest levels in Cascade beer; its varietal specificity and contributions to the characteristics of Cascade have been reported in the Chapter 2. Ethyl 4-methylpentanoate, which influences hoppy and citrus aromas in beer as described in the Chapter 2 and by Fritsch *et al.* [33], was present at the lowest levels in Simcoe. These results also support the hypothesis that 4MMP content affect on the total hop aroma.

Table 3-3. Contents of hop-derived main odorants in beer hopped with 11 cultivars.

	Difference threshold value in beer ^c	US- SIM (2005)	US- SUM (2006)	US- APO (2006)	US- MIL (2006)	US- CAS (2006)	US- WIL (2006)	GE- MAG (2006)	GE- TAU (2006)	GE- HER (2005)	CZ- SAZ (2005)	UK- FGL (2005)	Un- hopped	CV ^f (%)
4MMP (ng/L)	1.5	183.8	116.4	109.2	n.d.	16.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d. ^b	4.0
myrcene (µg/L)	9.5	28.3	57.7	37.5	37.3	21.4	11.9	41.6	89.2	11.4	12.9	5.47	n.d.	7.4
linalool (µg/L) (ee %)	1.0 ^d , 1.7 ^e	95.5 (85.2)	87.9 (85.9)	73.4 (85.4)	84.7 (89.5)	56.9 (87.6)	64.6 (89.2)	43.1 (87.1)	167.0 (92.2)	80.8 (91.9)	62.5 (87.4)	35.6 (90.1)	n.d.	4.3
geraniol (µg/L)	4.0	58.5	59.1	33.3	23.2	82.8	11.7	22.3	9.49	6.14	11.5	4.68	n.d.	5.7
ethyl 2-methyl -butanoate (µg/L) (ee %)	1.2 ^e	2.04 (-93.9)	5.38 (-97.5)	9.78 (-97.7)	2.75 (-97.5)	0.80 (-92.2)	1.01 (-91.8)	3.80 (-97.1)	2.61 (-96.7)	0.72 (-89.1)	0.80 (-85.5)	0.75 (-91.1)	0.09	7.5
ethyl 4-methyl -pentanoate (µg/L)	1.0	0.60	2.39	2.23	1.93	0.41	0.35	1.97	1.52	1.07	1.16	0.43	0.09	4.3

n.d. : not detected. ^a Mean values of duplicated analyses. ^b Concentration beneath the detection limit in beer (4MMP:< 4.0 ng/L, myrcene:< 0.001 µg/L, linalool:< 0.05 µg/L, geraniol:< 0.10 µg/L), where the height of the signal was 3-fold that of noise. ^c Difference threshold value (µg/L) in beer. ^d The value was determined by using the (*R*)-isomer. ^e The value was determined by using the racemate. ^f Coefficient of variation (%)

3-3-4. Comparison of 4MMP Content of Hop Pellets Grown in Different Regions. The concentrations of 4MMP in 17 hop pellet cultivars (**Figure 3-7a**) were determined using Dowex 1, *p*HMB, and MD-GC-MS with a detection limit of 1.19 µg/kg pellet; the height of the signal was three-fold greater than that of noise. The mean values of duplicate analyses are shown in **Figure 3-7a**.

4MMP was detected only in the hop pellets of USA, Australian and New Zealand cultivars. The highest content of 4MMP was observed in the cultivars, 2005 Simcoe (111.5 µg/kg) and Summit (88.2 µg/kg), followed by Apollo (61.4 µg/kg), Topaz (48.3 µg/kg), Cascade (11.1 µg/kg), USA Perle (4.61 µg/kg) and Pacific Gem (2.51 µg/kg). Genetic effects on the high concentration of 4MMP were, by necessity, considered as a reason for the difference in the content. However, even within the same cultivar such as Perle or Nugget, 4MMP was detected only in USA rather than German hop pellets. This is likely to be caused by the European use of copper sulfate (Bordeaux mixture) for protection against downy mildew. Thiols, that contain sulfhydryl group, have been reported to conjugate with copper ion [107, 109], and the wine made from grapes treated with Bordeaux mixture was found to lose its rich aroma [55].

In the divalent metal, copper ion is termed as soft metal ion [109], which has large acceptor atoms of relatively low positive charge, low electronegativity, and contain unshared pairs of electrons in their valence shells. Sulfhydryl group is termed as soft ligands with donor atoms of low electronegativity and high polarisability holding their valence electrons loosely. Soft metal ions preferentially bond to soft ligands [109]. **Figure 3-7b** shows the divalent metal ions in hop pellets as measured by ICP-MS. The European cultivars had extremely high copper ion contents, while those of the other divalent metal ions were similar among the different cultivars. These results imply that high concentrations of copper ions decrease the 4MMP content. And furthermore it is likely that undesirable pungent onion-like aroma in specific cultivars is also controlled by the use of copper sulfate.

Even within identical cultivars with similar copper ion and α -acid contents, the 4MMP content could vary between harvest year as in Simcoe hop pellets; the value for the 2006 crop was only one-fifth of that for the 2005 crop (**Figure 3-7a**). Further research is planned to investigation the reasons for this variation.

In conclusion, it was indicated that the copper ion content could be of particular relevance to the 4MMP content, as well as a genetic effect on the high content. Future work will include field tests to reveal the effects of copper on thiol contents in hop cones.

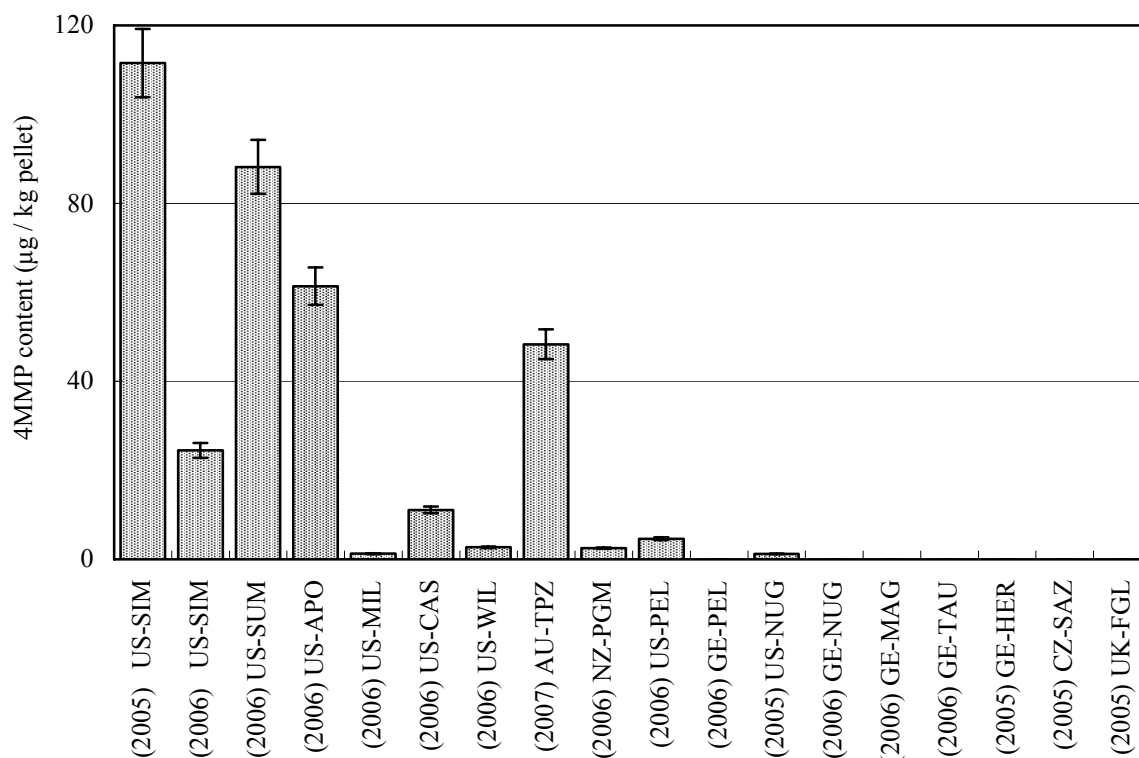


Figure 3-7a. 4-mercapto-4-methylpentan-2-one content in hop pellets

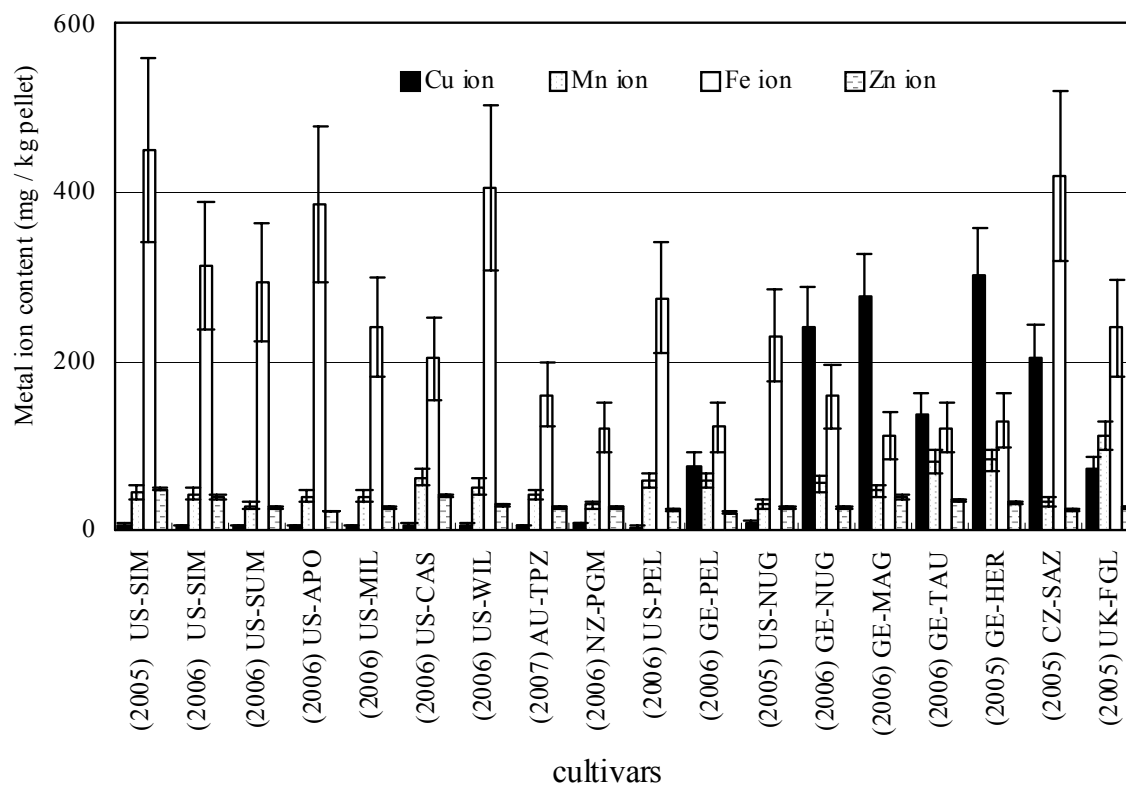


Figure 3-7b. 4-mercapto-4-methylpentan-2-one and copper ions contents in hop pellets.

Chapter 4

Behaviors of 3-Mercaptohexan-1-ol, 3-Mercaptohexyl Acetate during Brewing Processes

4-1. INTRODUCTION

To determine the components that contribute to the sensory hop aroma characteristics of beer, the author identified several hop-derived potent odorants, including thiols, that persisted even after fermentation in the Chapter 2.

In the Chapter 3, the author investigated the hop-derived thiols in beers hopped with cultivars from the USA and Europe, using an extraction method involving SAFE, a strongly basic anion-exchanger resin (Dowex 1), and *p*HMB. Seven odorants that contributed to the blackcurrant-like aroma, which is often attributed to the presence of thiols, were detected by GC-O analysis. Three of these seven odorants were identified as 4MMP, 3MH, and 3MHA, by comparing their mass spectra, RIs, and odor qualities with those of authentic compounds on both DB-WAX and HP-5 capillary columns, by GC-O and GC-MS. In the Chapter 3, the contribution of 4MMP to the aroma in beer hopped with USA cultivars were studied.

Although the thiols in beer seems to affect the overall flavors due to their extremely low threshold value, the behaviors of hop-derived 4MMP, 3MH and 3MHA have not previously been examined. 3MH and 3MHA were initially identified in passion fruit [27]. These volatile thiols have also been identified in Sauvignon Blanc wines [98]. Tominaga *et al.* reported the effects of 4MMP, 3MH, and 3MHA on wine flavors [18, 99, 102]. Vermeulen *et al.* [108] reported the presence of 3MH in fresh lager beers.

The current study investigated the behaviors of 3MH and 3MHA during brewing processes in order to retain these thiols in beers, and establish their contributions to the distinctive beer aroma.

4-2. MATERIALS AND METHODS

4-2-1. Reagents. Dowex[®] (1 × 2, Cl(−)-form, strongly basic, 50–100 mesh; CAS no. 69011-19-4) was obtained from Sigma-Aldrich (St Louis, MO). *p*-Hydroxymercuribenzoic acid sodium salt (CAS no. 138-85-2) was purchased from Acros Organics (Morris Plains, NJ). 4-mercapto-4-methylpentan-2-one (CAS no. 19872-52-7) and 4-methoxy-2-methyl-2-mercaptobutane were obtained from San-Ei Gen FFI, Inc. (Osaka, Japan). *tert*-Butyl-4-methoxyphenol (CAS no. 25013-16-5) and L-cysteine hydrochloride monohydrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tris(hydroxymethyl)aminomethane (CAS no. 77-86-1), HNO₃ for ICP-MS analysis, yttrium and indium for atomic-absorption analysis, and copper granules were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 3-mercaptohexan-1-ol (CAS no. 51755-83-0) was purchased from Avocado Research Chemicals Ltd. (Heysham, UK). 3-mercaptohexyl acetate (CAS no. 136954-20-6) was purchased from Atlantic Research Chemicals Ltd. (Bude, UK).

4-2-2. Hops. The hop cultivars Simcoe (2005 and 2006 crops, USA) and Topaz (2007 crop, Australia) were purchased from Yakima Chief (Sunnyside, WA) and Hop Products Australia (Tasmania, Australia), respectively. Hersbrucker (2005 crop, Germany), Saazer (2005 crop, Czech Republic), Fuggle (2005 crop, UK), and Perle (2006 crop, Germany) were purchased from Joh. Barth & Sohn GmbH & Co. (Nuremberg, Germany). Summit (2006 crop, USA), Millennium (2006 crop, USA), and Nugget (2005 crop, USA) were purchased from John I. Haas, Inc. (Yakima, WA). Apollo (2006 crop, USA), Cascade (2006 crops, USA), Willamette (2006 crop, USA), and Perle (2006 crop, USA) were purchased from S. S. Steiner, Inc. (New York, NY). Magnum (2006 crop, Germany), Taurus (2006 crop, Germany), Nugget (2006 crop, Germany), and Pacific Gem (2006 crop, New Zealand) were purchased from Simon H. Steiner, Hopfen, GmbH (Mainburg, Germany).

4-2-3. Brewing Processes. Pellets of the hop cultivars; Simcoe, Summit, Apollo, Willamette and Fuggle were used in the brewing processes. Hopped and unhopped beers were brewed independently in 20 L volumes as described in the Chapter 3.

To trace the changes in the 3MH and 4MMP concentrations during wort boiling, 50 g Simcoe pellets was added to 20 L wort at the beginning of the brewing process, and the mixture was then either boiled at an intensity of 8 %, or kept at 90 °C.

4-2-4. Determination of Difference Threshold Values. The difference threshold values of 3MHA were determined using the method of the ASBC, as described in the Chapter 2.

4-2-5. Isolation of Volatile Thiols. Volatile thiols were isolated from the hop pellets, wort, and beer using a strongly basic anion-exchanger resin (Dowex 1) and *p*HMB with elution columns, as described in the Chapter 3.

4-2-6. Quantification of Thiols by MD-GC-MS. The 3MH, and 3MHA contents in the wort and beer were quantified using MD-GC-MS as described in the Chapter 3.

4-2-7. Quantification of Divalent Metal Ions. Divalent metal ions were quantified as described in the Chapter 3, using ICP-MS (Agilent 7500c). Prior to the ICP-MS analysis, the samples were digested in closed PTFE vessels using a Multiwave 3000 microwave sample-digestion system (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA).

4-2-8. Lead-Conductance Value of Hop Pellets. The lead-conductance values of the hops were determined using the EBC 7.4 method [30].

4-3. RESULTS AND DISCUSSION

4-3-1. 3MH and 3MHA Concentrations in Hop Pellets. In hop pellets, 3MH was detected in both USA and European cultivars (**Figure 4-1**), while 3MHA content was below the detection limit. The results are presented as the mean values of duplicated analyses with the coefficients of variation: 3MH = 23.2 %; 3MHA = 5.4 %, and detection limits; 3MH = < 1.50, 3MHA = < 0.17 µg/kg pellet (where the height of the signal was three-fold that of the noise). The results showed that the 3MH concentration differed between cultivars in the range from 10 to 120 µg/kg pellet. The highest 3MH content was detected in the hop pellets of the cultivar Simcoe (2006; USA), followed by Topaz (Australia), Fuggle (UK), and Cascade (USA). In the Chapter 3, the author reported that the content of 4MMP depended on the growing region as well as the cultivars. 4MMP was detected in the hop pellets of cultivars from the USA, Australia, and New Zealand, but not from Europe. It was suggested that this phenomenon could be caused not only by a genetic effect, but also by the use of copper sulfate (Bordeaux mixture) in Europe to protect against downy mildew.

The contents of 3MH did not correlate with those of 4MMP. The reasons for little or no effects of growing regions on 3MH content were unclear. The effect of copper was examined by the excess addition of copper granules to beer. Copper granules (50 g) were added to beer (250 ml) hopped with Apollo and Simcoe, and then left overnight at 4 °C. As shown in **Figure 4-2**, the 4MMP content decreased by 50% following the addition of copper granules, whereas the 3MH and 3MHA contents remained unchanged. These results confirmed that 4MMP is adsorbed by copper ions, and is more susceptible to copper ions than 3MH. These findings also corroborate the fact that the 3MH content was not affected by growing regions, copper content (**Figure 4-1**).

Even within a single cultivar, the 3MH content differed greatly between crop years (**Figure 4-1**). This was particularly evident in Simcoe hop pellets, in which the 3MH content of the 2006 crop was twice that of the 2005 crop, while the α -acid and copper ions were relatively unchanged.

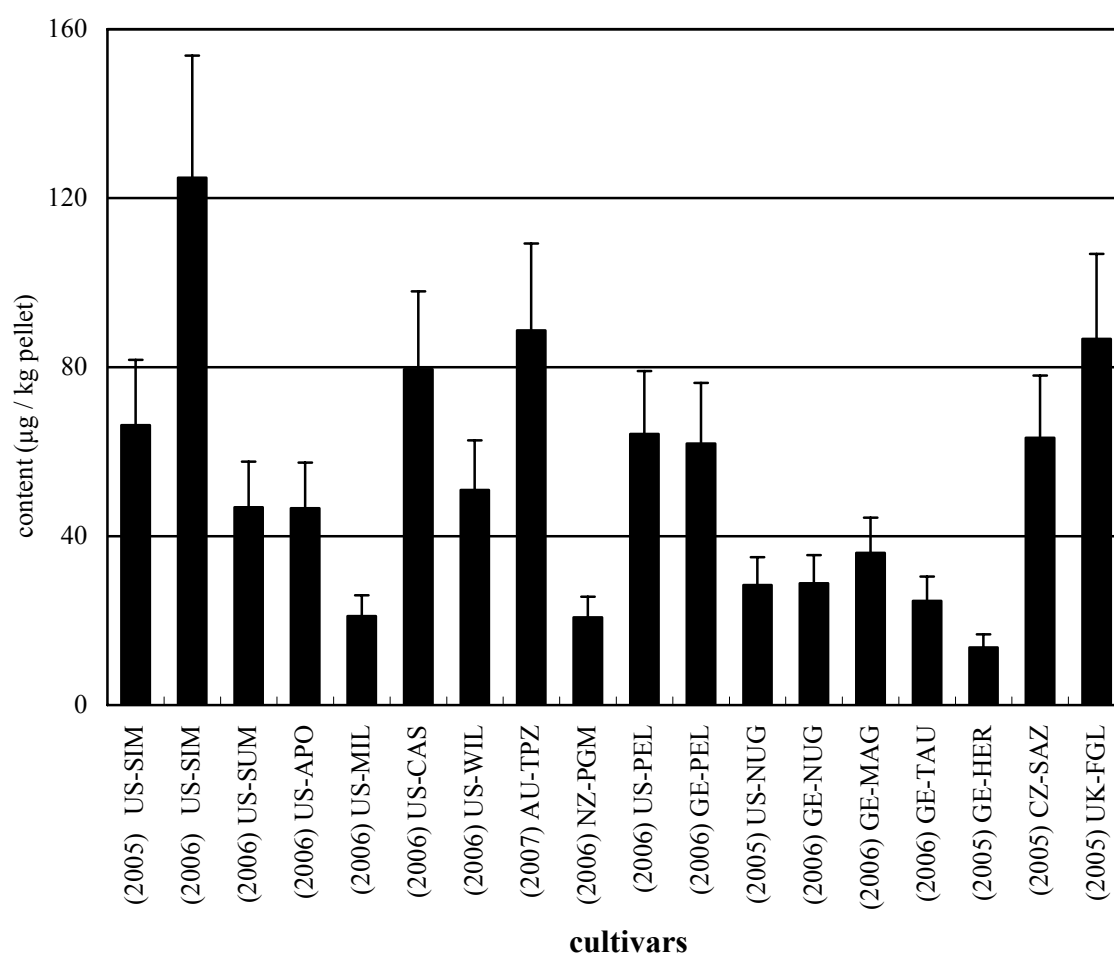


Figure 4-1. 3-mercaptohexan-1-ol contents in hop pellets of different cultivars.

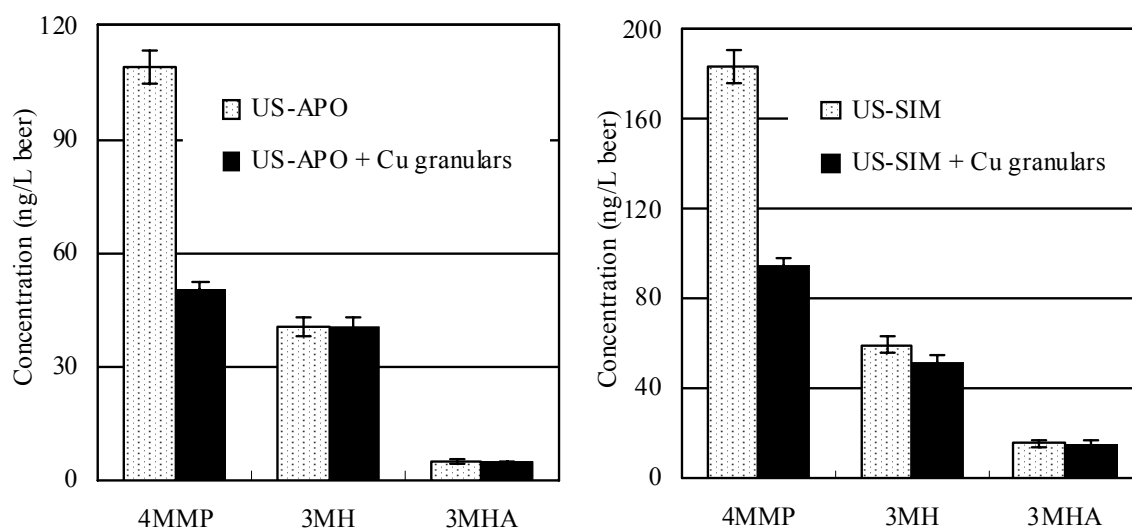


Figure 4-2. Comparison of 4-mercapto-4-methylpentan-2-one, 3-mercaptohexan-1-ol, and 3-mercaptohexyl acetate contents in beers hopped with Apollo and Simcoe, with and without added copper granules.

4-3-2. 3MH Contents in Beers. The 3MH contents in worts and beers hopped with the 5 cultivars are shown in **Figure 4-3a**. The results are presented as the mean values of duplicated analyses with the coefficients of variation: 3MH = 7.0% in wort and 6.3% in beer; 3MHA = 4.9% in wort and 8.4% in beer. The detection limits were 3MH = < 12.0 in wort and < 10.2 ng/L in beer; 3MHA = < 4.4 in wort and < 3.9 ng/L in beer, where the height of the signal was three-fold that of the noise. In order to investigate the aroma quality of hop-derived components (which can survive even after fermentation) without the temperature effects on the thiols with low boiling temperature, the hops were added after the wort-cooling process as described in the Chapter 3.

3MH was detected in the beers of all cultivars in the range from 35- 59 ng/L, highest in Simcoe beer, followed by Willamette and Apollo beers. The contents were close to or below difference threshold value (55 ng/L). It indicated that the contributions of 3MH to beer characteristics were much smaller than those of 4MMP, which was included at extremely higher content than threshold value in beers hopped with some cultivars as described in the Chapter 3.

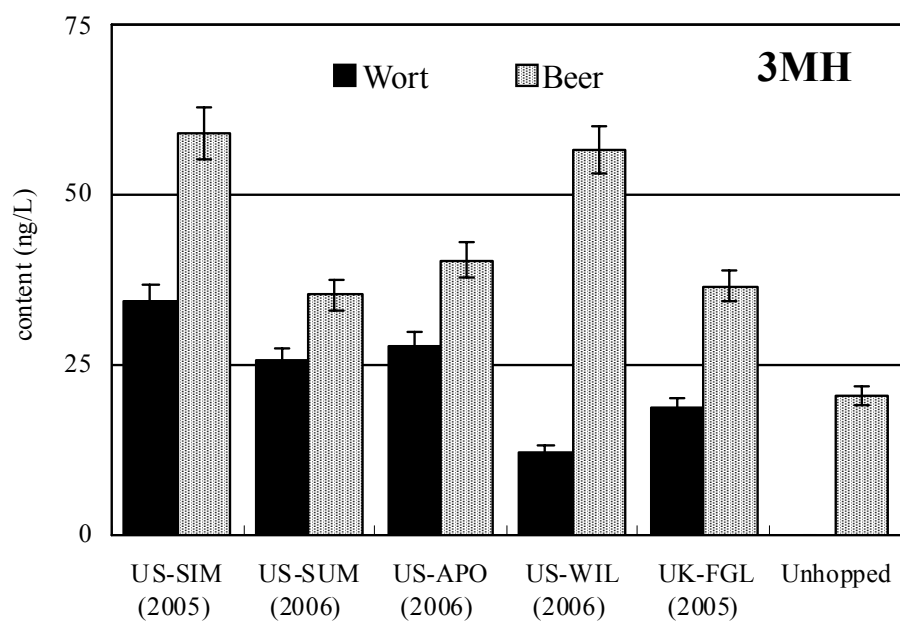


Figure 4-3a. 3-mercaptohexan-1-ol contents in beers and worts.

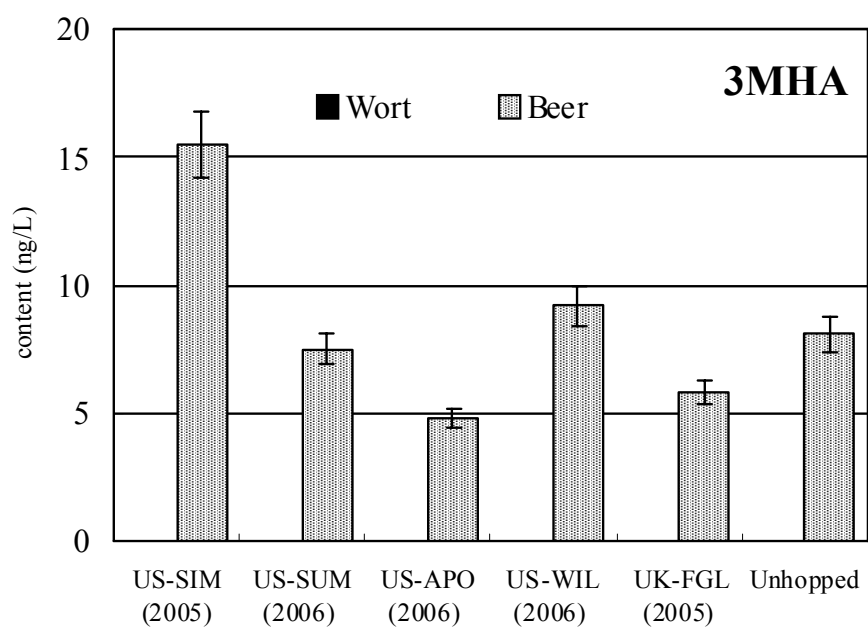


Figure 4-3b. 3-mercaptohexyl acetate contents in beers and worts.

4-3-3. Behavior of 3MH and 3MHA during the Brewing Process. During the wort-boiling process, the 4MMP content decreased by 91% after boiling at 100 °C for 60 min, and by 23% after heating at

90 °C (**Figure 4-4a**). Interestingly, with hopped wort, the 3MH content increased (**Figure 4-4b**) at a faster rate at 100 °C (at which the content increased three-fold) than at 90 °C. Furthermore, 3MH was not detected in unhopped wort. These results indicated that 3MH was thermally formed during the wort-boiling process from precursors that existed in the hops. During fermentation, the 3MH (**Figure 4-5a**) and 4MMP contents increased, and peaked at early stages of fermentation (**Figure 4-5a**), as previously observed in wine [95].

While 4MMP was not detected in both unhopped wort and beer as described in the Chapter 3, measurable amounts of 3MH was detected in hop pellets of cultivars (**Figure 4-1**) and even in unhopped beer, but not in unhopped wort (**Figure 4-3a**), indicating that malt and hops both contain 3MH precursors.

Previous studies [95, 100] have reported on cysteine conjugate precursors that lead to the release of 3MH and 4MMP during fermentation processes. Vermeulen *et al.* [108] proposed the synthetic pathways of 3MH from precursors that they are formed by the biolysis of cysteine conjugate, or by Michael addition of H₂S on (*E*)-2-hexenal followed by a reduction. In the present study, it was unclear why 3MH increased or released into wort by boiling (**Figure 4-4b**). Further researches are required to clarify the formation of 3MH from precursors, such as cysteine conjugate or glycosides.

It was shown that 3MHA was not detected in the worts (**Figure 4-3b**) and was newly synthesized (**Figure 4-5b**) from 3MH by the action of a yeast ester-forming alcohol acetyltransferase [95, 96]. Yeasts with high capability of converting 3MH into 3MHA were studied in the former literature [95, 96]. The difference threshold value of 3MHA was newly determined in the current study as 5.0 ng/L, which was lower than that of 3MH (55 ng/L) [Chapter 2]. Therefore, selecting yeast strains with a higher capacity of bioconversion of 3MH into 3MHA is a useful strategy to increase the aroma impact.

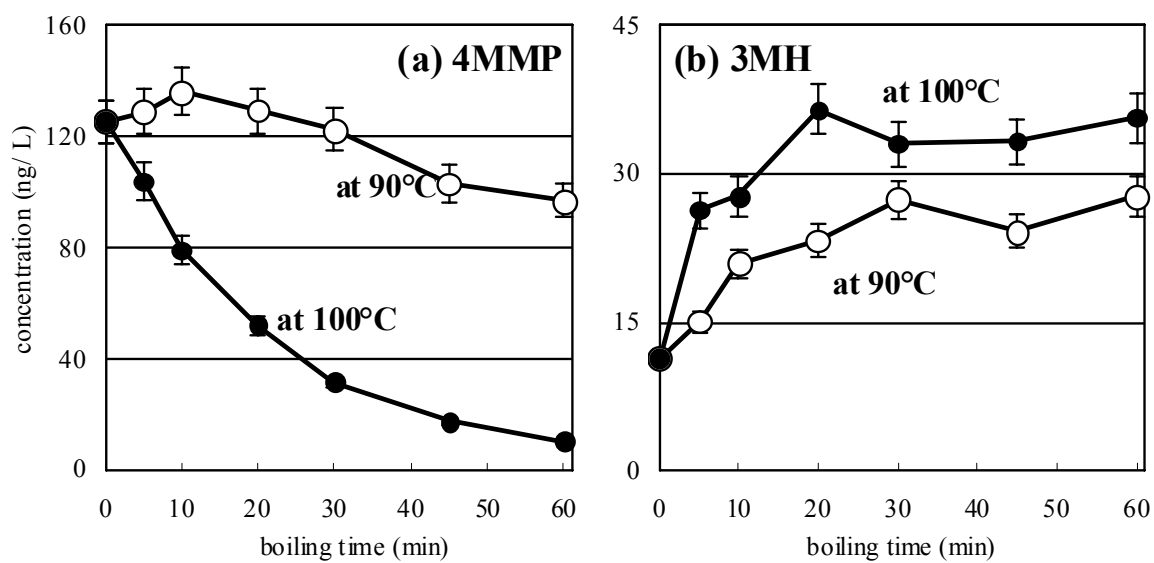


Figure 4-4. Changes in 4-mercapto-4-methylpentan-2-one(a) and 3-mercaptohexan-1-ol (b) concentration of wort hopped with Simcoe during boiling process.

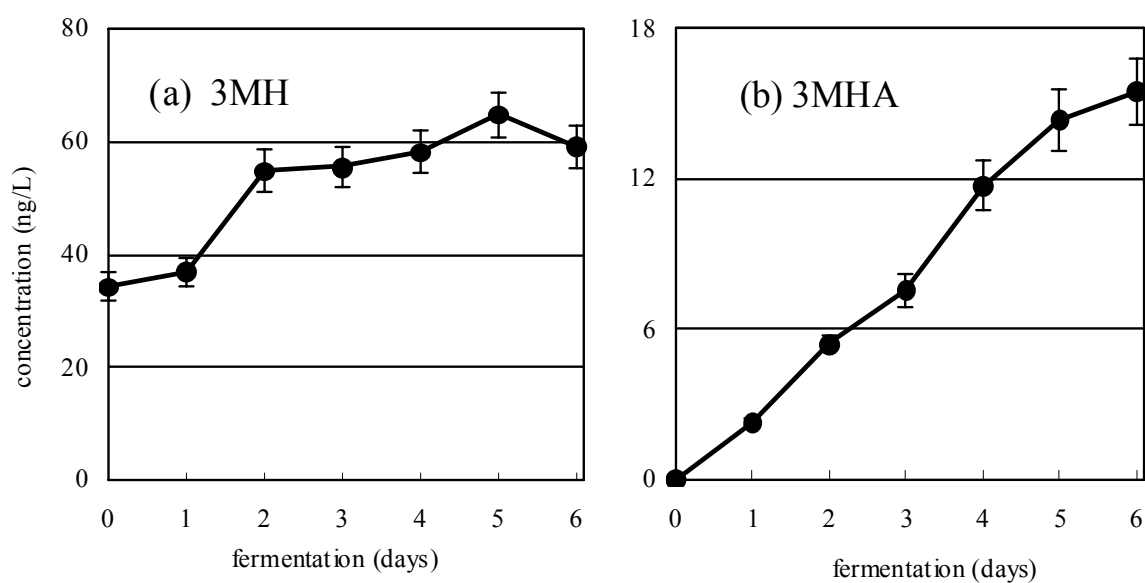


Figure 4-5. Changes in 3-mercaptohexan-1-ol (a) and 3-mercaptohexyl acetate (b) concentrations of wort hopped with Simcoe during fermentation.

Chapter 5

Hop-Derived Odorants Increased in the Beer Hopped with Aged Hops

5-1. INTRODUCTION

α -acids and β -acids in hop cones start being broken down into short chain acids, by the influences of oxygen, temperature and humidity. These short chain acids remain in the beer, therefore, the qualities of bitterness hopped with aged hops are described in some reports [57, 74], however, excessive break-down of bitter substances yields cheesy smell in beer derived from the acids. The moderate aging of hops prior to the brewing provides citrus and floral characteristics to beer [22, 60], while the contributors to the characteristics had not been studied in detail.

In this chapter, the odorants that comprise the characteristics of the aroma in beer hopped with aged hops were investigated, by examining the concentrations of terpenoids and esters, which were described in the Chapter 2.

5-2. MATERIALS AND METHODS

5-2-1. Brewing Processes. A bitter cultivar of hop pellets (11.5% α -acid hop pellets from the 2005 crop) was used in this chapter. Beers were brewed independently using hop pellets that had either been aged by storing at 40 °C for 30 days or had been cold-stored at 4° C. During the brewing process, 20 L wort was hopped with 16 g hop pellets either after the wort cooling process or at the beginning of the boiling process. Static fermentations were carried out in 5 L stainless-steel tall tubes, modified from 2 L EBC tall tubes, which were equipped with precise pressure and temperature control mechanisms (**Figure 2-1**). The yeast used for the fermentation was cultured in unhopped wort for 3 days, in order to wash out the hop-derived components. The yeasts were recovered by centrifugation, and were pitched at a rate of 20 million cells per milliliter. A 4.5-L sample of each type of wort (11.5 °P) was fermented at 12.0 °C for 8 days, and any yeast that had settled at the bottom of the tank was removed. After a maturation step carried out at 12.0 °C for 4 days, the beers were cooled to 0 °C for 5 days, and then centrifuged in order to obtain the finished beers.

5-2-2. Sensory Evaluation. Flavor-profile analyses were performed by seven trained sensory panelists on the beers hopped with the aged hops and the cold-stored hops. The members of the sensory panel were asked to evaluate the intensity of the pellet-like aroma, the citrus aroma, and the total hop aroma. The odor intensities were rated on the following scale (with intervals of 0.5 points): 0 = not perceivable; 1 = weak; 2 = normal; 3 = strong; and 4 = very strong. The characteristics of the hops were compared by calculating the mean intensity values of the scores.

5-2-3. Quantifications of Ethyl 4-methylpentanoate and MBT. Ethyl 4-methylpentanoate and MBT were quantified, as described in the Chapter 3, using the large volume dynamic headspace method with GC-MS and the Entech 7100A system (Entech, USA).

5-2-4. Quantification of Other Volatiles. The linalool, geraniol, and β -ionone concentrations in the worts and beers were quantified using the SBSE method with β -damascone as an internal standard, as described in the Chapter 1. The amounts of ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, (*Z*)-3-hexen-1-ol, 2-phenylethyl 3-methylbutanoate, and 4-(4-hydroxyphenyl)-2-butanone in the beers were measured by the liquid-extraction method with dichloromethane using (–)-borneol and (*Z*)-3-hepten-1-ol as internal standards, as described in the Chapter 1. All data are shown as the mean values of duplicated analysis.

5-2-5. Determination of ee Values. The enantiomeric ratios of linalool and ethyl 2-methylbutanoate were determined as described Chapter 3.

5-3. RESULTS AND DISCUSSION

5-3-1. Characteristics of the Beers Hopped with Aged Hops. The specifications of the beers brewed for use in this chapter are shown in **Table 5-1**. Some of the components were assumed to be derived from the deterioration of α -acids; the hop cultivar with the higher α -acid content was thus used to clearly demonstrate increases in the concentrations of the components studied.

During the brewing of beers C and D, hops were added only after the wort cooling, in order to clearly distinguish the hop aroma characteristics. The intensities of the pellet-like aroma, citrus aroma, and total hop aroma, and the comments made on the characteristics of beers C and D, are detailed in **Table 5-2**. Beer C, hopped with cold-stored hops, was characterized by hop pellet-like, resinous, green, and floral

notes, and the intensity of the hop pellet-like aroma was relatively strong. Beer D, hopped with aged hop pellets, was described as having citrus and sweet aromas. The intensities of the citrus aroma and the total hop aroma were slightly stronger in beer D.

During the brewing of beers A and B, the hops were added at the beginning of the boiling process in order to isomerize α -acids and to allow the formation of trub. Beer B was characterized by a smooth bitterness with an afterglow, while beer A was characterized by astringency and coarse bitterness, although both did not have any aroma characteristics.

Table 5-1. Brewing specifications of the beers used in the current chapter.

Beer type	A	B	C	D
Storage conditions for hop pellets	4 °C	40 °C for 30 days	4 °C	40 °C for 30 days
Lead conductance value of α -acids in hop pellets (%) ^(a)	11.5	4.1	11.5	4.1
Non-isohumulone bittering compounds in hop pellets (%) ^(b)	13.5	29.4	13.5	29.4
Time at which the hops were added	At the beginning of boiling only	At the beginning of boiling only	After the wort cooling only	After the wort cooling only
Bitterness units (BU) of the beer ^(c)	31	30	7	17

^a Determined using the EBC 7.4 method. ^b Determined using the EBC 7.7 method. ^c Determined using the EBC 9.6 method.

Table 5-2. Aroma profiles of beers C and D.

Beer type	C	D
Total hop aroma (intensity)	2.6	2.7
Citrus aroma (intensity)	2.2	2.4
Hop pellet-like aroma (intensity)	3.1	2.4
Comments on the hop aroma characteristics of the beer (frequency of comments)	Hop pellet-like (3) Resinous (2) Floral (2) Green (1)	Citrus (2) Muscat-like (2) Grape (1) Sweet (1)

5-3-2. Odorants with Increased Concentrations in Beers Using Aged Hops. In the Chapter 2, the author identified hop-derived odor-active components. **Table 2-2** shows the RIs on the DB-WAX column, the compounds identified, the difference threshold values, and the odor qualities detected by CharmAnalysis™. Newly synthesized components produced by the hop-aging process were not detected by the GC-O analysis of the odorants (data not shown). The changes in the concentrations of terpenoids and esters caused by hop aging, and their derivations, were examined.

In the beers hopped with aged hops, the increased concentrations were observed for low threshold value citrus components (ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 4-methylpentanoate, and 4-(4-hydroxyphenyl)-2-butanone). In particular, the concentrations of ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, and 4-(4-hydroxyphenyl)-2-butanone were close to or above the threshold values.

By contrast, significantly decreased concentrations of green, hop pellet-like, and resinous components (myrcene and (*Z*)-3-hexen-1-ol) were detected in beers hopped with aged hops. The extremely higher content of myrcene in the beer C decreased below the threshold value in the beer D. In current chapter, the difference threshold value in beer, 9.5 µg/L, was newly determined for myrcene, which was previously reported as a potent odorant in hop cone [14, 21, 89]. (*Z*)-3-hexen-1-ol can be generated easily by the reduction of aldehydes, such as (*Z*)-3-hexenal [40], during fermentation; these aldehydes were thus also assumed to decrease in concentration during the aging of hops.

Increased concentrations of MBT, β -ionone, and 2-phenylethyl-3-methylbutanoate were observed in the beers using aged hops, whereas the amounts of geraniol and ethyl 2-methylpropanoate were similar to those in the beers hopped with cold-stored hops.

The author proposed that changes in the balances of these components had a significant influence on the citrus characters and the total hop aroma intensities of the beers using aged hops, while the linalool content decreased.

The threshold value of linalool changes according to the isomer [90]. The value for the racemate linalool was newly determined here, and was higher than that for the (*R*)-isomer. As shown in **Table 5-3**, the ee values for linalool were similar in beers C and D, suggesting that racemization had not taken place, and that the aroma values for linalool remained constant throughout the hop aging process. By contrast, beers A and B had decreased ee values compared with beer C and D respectively. This implied that the (*R*)-isomer predominated in the hops, and that racemization had occurred during the boiling process, as previously suggested by Steinhaus [90].

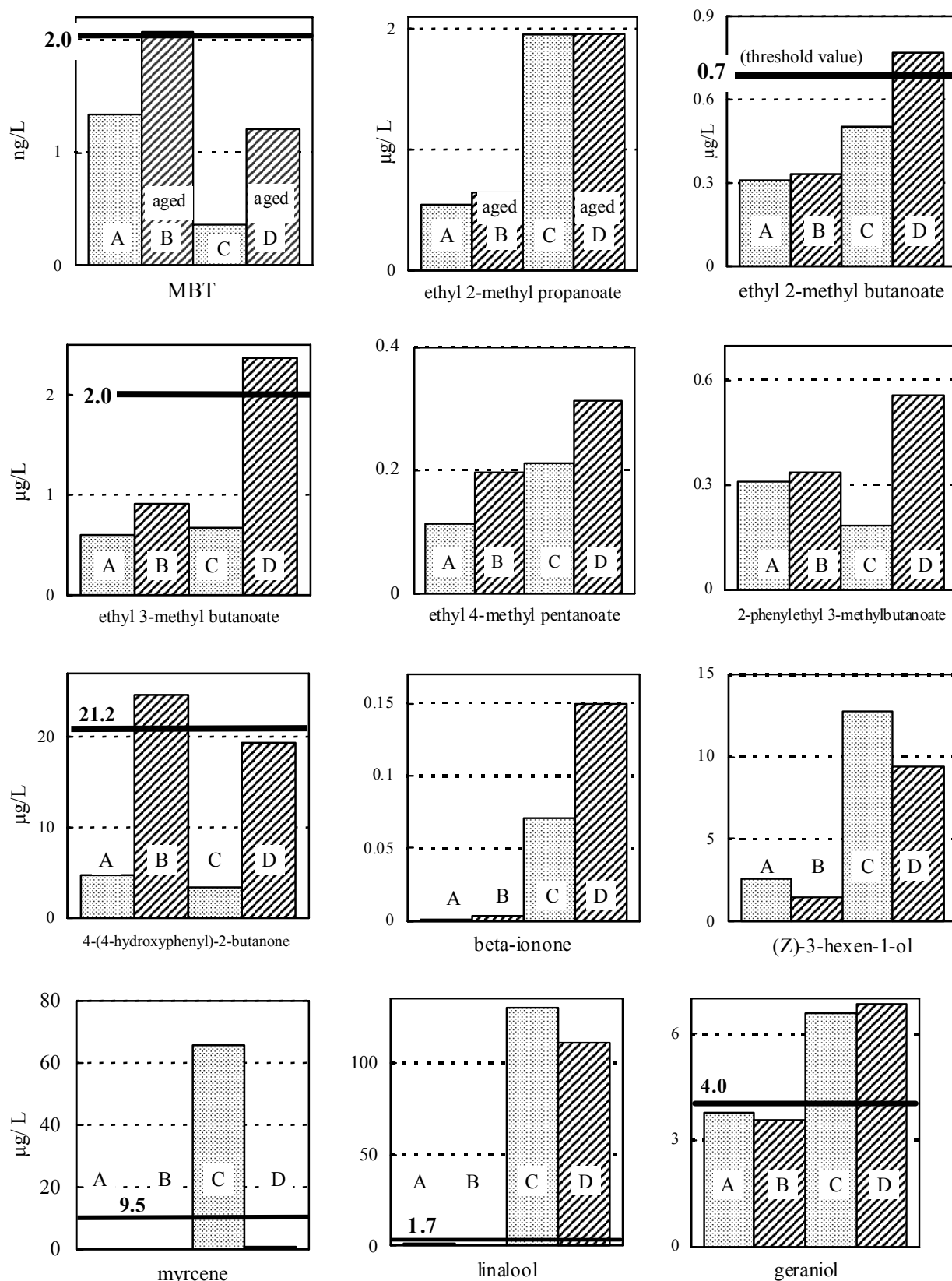


Figure 5-1. Concentrations of hop-derived components in beers hopped with aged hops and hops stored at 4 °C. The threshold values are indicated by the bold lines.

Table 5-3. The ee values of linalool and ethyl 2-methylbutanoate.

Beer type	A	B	C	D
Ethyl 2-methylbutanoate	-32.6	-36.9	-81.9	-63.4
Linalool	38.4	28.8	91.6	90.5

5-3-3. Hypothetical Synthetic Pathway of Odorants. Increases in the concentrations of MBT and some esters were observed throughout hop aging. Similar degradations were supposed in formations of both MBT precursors and short chain fatty acids.

In the Chapter 2, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 4-methylpentanoate, 2-phenylethyl-3-methylbutanoate, and 4-(4-hydroxyphenyl)-2-butanone were either detected in small amounts or not detected before fermentation. In our current chapter, increased concentrations of the esters were detected in the beers hopped with aged hops. Based on our results, these components were assumed to be formed by the esterification of short-chain fatty acids that were derived from the oxidation or degradation of hop components. One hypothetical synthetic pathway for these components is from humulone and lupulone, which are principal hop components.

Compounds derived from the oxidation or degradation of hop components are known as “non-isohumulone bittering compounds” [57] or the “S-fraction” [74]. Beer B had a relatively high S-fraction content (**Table 5-1**) and relatively low iso- α -acid ratios, considering from its non-isohumulone bittering compounds, lead conductance value in hop pellets, and BU value.

3-methylbutanoic acid, 2-methylpropanoic acid, 2-methylbutanoic acid, and 4-methylpentanoic acid appeared to be generated by the degradation of normal, co-, ad-, and pre-humulone or lupulone compounds, respectively (**Figure 5-2**). Reduced concentrations of esters were detected in beers A and B (**Figure 5-1**), in which the hops were added at the beginning of the boiling process; these short-chain acids were thus assumed to be partly trapped in the trub or evaporated during boiling.

A relatively high concentration of β -ionone was detected in these beers B and D. In nature, this compound is thought to originate from β -carotene, and can be produced by direct oxygenation during storage in air [6] or by aerobic fermentation [81, 82].

Interestingly, the highest concentration of MBT was detected in beer B, which was hopped with aged hops at the beginning of the boiling stage, in spite its iso- α -acid content was lower than those of beer A. Therefore some precursors of MBT were pre-formed during the aging of the hop pellets. However, in the present study, the author was unable to determine whether the MBT was generated during fermentation or during the boiling process.

Based on these findings, the author proposed that hop aging induces the fragmentation of α -acids at several different points to generate components such as short-chain acids and MBT precursors. Further investigations will be necessary to reveal the mechanisms of these pathways.

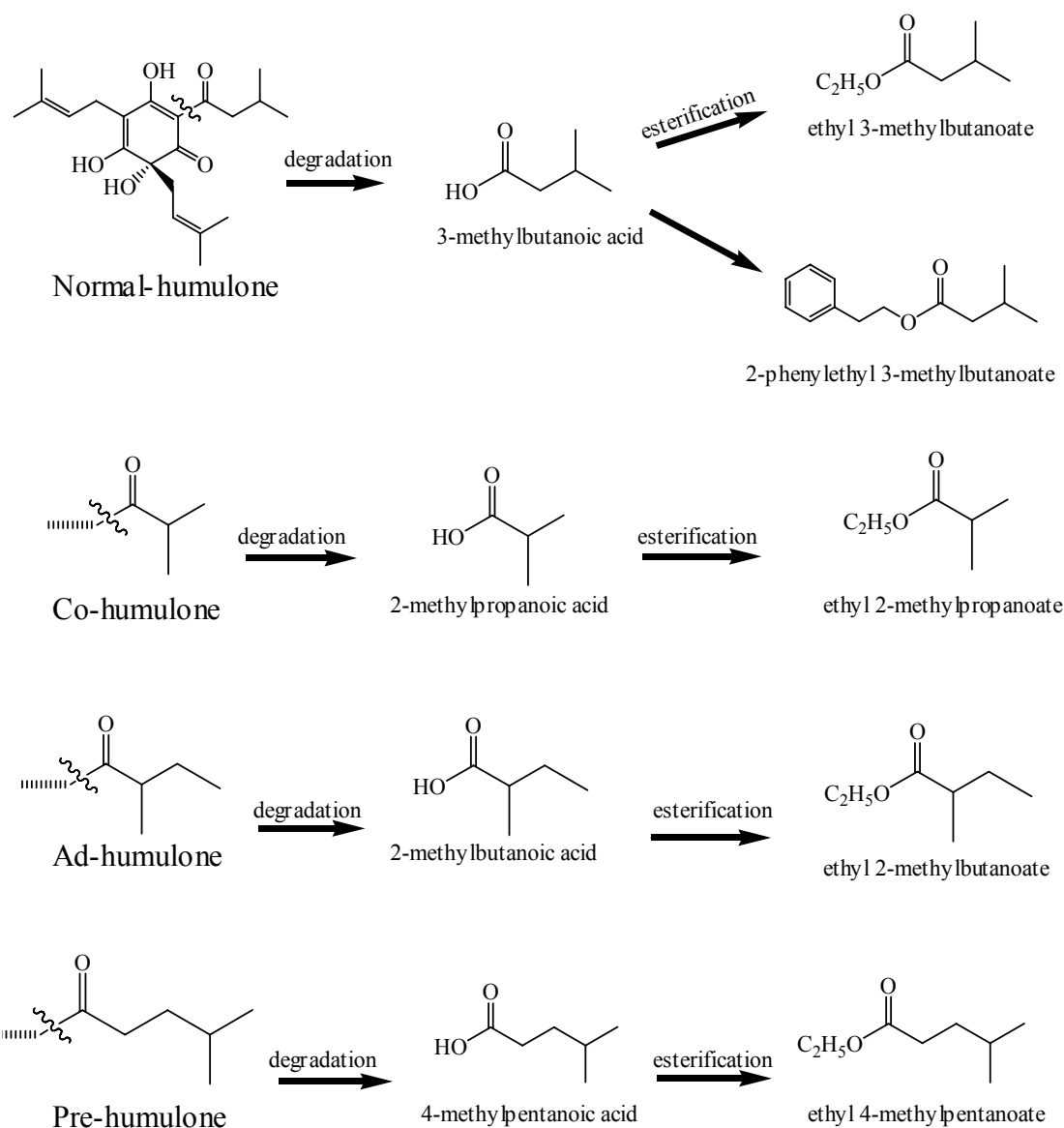


Figure 5-2. Hypothetical synthetic pathway of esters from α -acids

Summary (English)

Chapter 1. Between 50 and 80 % of the hop essential oil is composed of hydrocarbons, which are mainly terpenoids such as myrcene, caryophyllene, and humulene. The terpenoids have been examined in many researches as the main contributors to the hop aroma of beer. The analysis of odorants in beer requires various pretreatments. Moreover, matrices such as proteins, polyphenols, and fatty acids impede the extraction of terpenoids that are present in trace amounts. Raw hops or large volumes of beers with rich hop aromas have thus been required in order to extract sufficient quantities of hop-derived terpenoids for analysis. The current research examined the analysis of terpenoids using the SBSE method. This approach utilizes magnetic stir bars coated with glass and PDMS. The odorants, including terpenoids, adsorb to the PDMS when the bar is stirred in beer or wort samples. The adsorbed odorants are released by heating the stir bar in the inlet of GC-MS apparatus, and the odorants are then injected into the GC-MS column. This method makes it possible to analyze accurately trace amounts of terpenoids. The terpenoid contents of wort and beer with strong hop aromas were analyzed using this method. The results revealed that the concentrations of most terpenoids, except linalool and geraniol, were notably lower than their threshold values, and indicated the existence of trace amounts of many odorants contributing to the hop aroma characteristics.

Chapter 2. Strongly hopped beers, produced using approximately fivefold greater amounts of hops than normal, were compared with unhopped beers by GC-O and sensory evaluation, in order to reveal the odor-active components comprising the beer hop aromas. A sensory-evaluation method was established to describe the characteristics of different cultivars. The results of the sensory evaluation indicated that the beers hopped with the Saazer cultivar had citrus and floral characteristics, those hopped with the Cascade cultivar had muscat and blackcurrant-like characteristics, and those hopped with Hersbrucker had a spicy aroma. The odorants contributing to these characteristics were investigated using a GC-O analysis of those extracted from the hopped and unhopped beers with dichloromethane. The results revealed 27 odorants that were hop-derived, which existed in the hopped beers but not the unhopped beers, and identified 19 components. Based on the intensity of each odorant in the GC-O analysis, the citrus and floral characteristics were attributed to esters, terpenoids, ketones, and thiols, and the green characteristic was attributed to aldehydes and alcohols. The contributors to the spicy characteristic were detected in the GC-O analysis, although the odorants were not identified. The odorant with a sulfhydryl group, 4MMP was identified as a contributor to the muscat and blackcurrant-like characteristic of the

Cascade cultivar.

Chapter 3. The previous chapter revealed the contributions of hop-derived thiols, such as 4MMP and 3MH, to the hop aroma characteristics. These thiols have extremely low threshold values, and their contributions to wine aromas have previously been reported. In the current chapter, the thiol contents of hopped beers were examined using a method that extracts them effectively with *p*HMB and a strongly basic anion-exchanger resin. This revealed that 4MMP contributed to the characteristics of the Cascade cultivar, and its content in the beers was around 15 ng/L. To establish the hop-derived muscat and black-currant-like characteristic in beers, the 4MMP contents of other cultivars were investigated. The results of a comparison of the 4MMP content among 17 cultivars revealed that it was present not only in the Cascade cultivar, but also in cultivars from the USA, New Zealand, and Australia, some of which contributed extremely strong fruity and black currant-like aromas to the beers. 4MMP was not detected in any of the European cultivars, which had relatively high contents of copper ions. European cultivars are treated with copper-containing fungicides (Bordeaux mixture) against mildew. A negative correlation between the 4MMP concentration and the copper-ion content of the hops was observed in the hop pellets. 4MMP easily binds to divalent metal ions. It was thus suggested that 4MMP loses its aroma by forming a bond with the copper ions in hop cones. The behaviors of 4MMP during the fermentation process were investigated. The 4MMP content was found to increase by 30% during fermentation; this suggested that most 4MMP exists freely in wort or hop pellets, with only small amounts (30%) being formed from precursors.

Chapter 4. The thiol 3MH has similar aroma characteristics to 4MMP. It has a threshold value of 55 ng/L, is found in beers at contents of around 50 ng/L, and is thought to contribute to the muscat and black-currant-like characteristic. In hops, 3MH was detected in cultivars from both the USA and Europe, at concentrations ranging from 10 to 120 µg/kg pellet, whereas 4MMP was not detected in the European cultivars. The addition of copper granules to the beers indicated that 4MMP was more susceptible than 3MH to binding with metal ions, which could explain the fact that the growing regions of the cultivars had little effect on the 3MH contents.

During the boiling process of hopped wort, the 4MMP content decreased, whereas the 3MH content increased at a faster rate at 100 °C than that at 90 °C. During fermentation, the 3MH and 4MMP contents increased, and they peaked at an early stage of the process. 3MH was also detected in measurable amounts in the hop pellets of all of the cultivars and in unhopped beer, but not in unhopped wort. This finding indicated that both malt and hops were a source of 3MH precursors. Little or no 3MHA was detected in the hop pellets. A portion of the 3MH was found to be converted during

fermentation to 3MHA, which had similar characteristics but a much lower threshold value (5.0 ng/L). The capacity to transform into 3MHA differ among yeast varieties. Selecting yeast strains with a high ability to convert thiols from 3MH into 3MHA is thus a useful strategy to increase the aroma impact.

Chapter 5. Moderate aging of hops prior to the brewing process provides a characteristic aroma to the beer. In this chapter, the contributors to these characteristics were investigated. Beers brewed with aged hops, which had been stored at 40 °C for 30 days, were evaluated in comparison to beers produced with hops that had been stored at 4 °C. The results of sensory evaluation revealed that the beers produced with aged hops had citrus/estery characteristics, while those produced using normal hops stored at 4 °C had green and hop pellet-like characteristics. With respect to the 27 hop-derived odorants that were identified in Chapter 2, the beers produced using aged hops contained decreased concentrations of green odorants, including myrcene and (*Z*)-3-hexen-1-ol, while the concentrations of MBT, β -ionone, and esters (ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 2-methylpropanoate, and ethyl 4-methylpentanoate) were increased. Based on the structures of these esters, it appeared that they were synthesized during the fermentation process from the substrate short-chain acids, which were generated by the oxidative degradations of humulone or lupulone. These esters existed at concentrations of 0.1 – 4.0 $\mu\text{g/L}$ in normal lager beer, and were increased by twofold to fourfold in the beers produced using aged hops. The changes in the ratios of these odorants might thus contribute to the citrus/estery characteristic of the beers produced with aged hops.

Conclusion. This research investigated the hop-derived odorants that comprise the beer hop aroma and their contributions to the characteristics of beers, in relation to different cultivars and hop usages, based on their sensory characteristics, contents, threshold values, and aroma characteristics. The knowledge obtained will be useful for manipulating and designing hop aroma quality.

In order to reveal the contributors to hop aroma characteristics in more detail, identification of the unknown components mentioned in the current work, and quantification of the components with extremely low threshold value, followed by aroma simulations recombining the odorants will be required in future research.

Summary (日本語)

1. 緒言

ホップはビールに特有の苦味と香りを付与するために用いられる。ビールの仕込み工程で、粉碎した麦芽と副原料を湯に加えて糖化させ、その糖化液を濾過したものが麦汁と呼ばれる。ホップは麦汁の煮沸工程中または煮沸後に加えられる。煮沸工程の初期にホップを添加すればビールに苦味を付与することができ、煮沸工程後半または工程後にホップを添加するとビールにホップ由来の香りを付与することができる。ビール用のホップとして 100 種類以上もの品種が世界中で栽培されており、品種、醸造工程中の添加方法を変えることによって、異なる質のホップ香気がビールに付与されることが経験上知られている。ビールの香り品質を設計するためには、ビールのホップ香気に寄与する成分を特定し、醸造工程中でそれらの成分をコントロールしていくことが欠かせない。醸造に使用されるホップ中の精油成分は 3% 以下であるが、その中には 450 種以上もの揮発性成分が含まれる。しかし、そのほとんどはビールには移行しないため、ビールに付与されるいわゆる“ホップ香”と、原料のホップそのものの香りとは全く異なっている。ビール中にはホップ以外の原料に由来する夾雑物質が多く含まれている。そのためビール中でホップ香気に寄与する成分を解析することは困難であり、これまで詳細な報告はなされていない。本研究では、ホップ由来の香気成分を解析し、特徴的な香りをビールに付与する方法について検討した。

2. ビール中のホップ由来テルペン類の解析

ホップの精油成分のうち 50~80% は炭化水素で占められており、その大部分はモノテルペン(C10)とセスキテルペン(C15)である。ビール中のテルペン類はホップのみに由来し、テルペン類がホップ香に対する寄与成分と考えられてきた。ビール中の香気成分の分析には多くの前処理が必要であるため、特に微量のテルペン類については、溶媒を用いた抽出法や固相カラムを用いる従来の抽出方法では検出されなかった。本報告では新たなテルペンの分析方法として Stir Bar Sorptive Extraction (SBSE) 法を検討した。SBSE 法ではポリジメチルシロキサンでコーティングされたガラス製の攪拌子を用い、この攪拌子をビールまたは麦汁中で 2 時間攪拌させることによってテルペン類を含む香気成分を攪拌子に吸着させる。この攪拌子を GC-MS 装置の注入口で加熱すると、香気成分が遊離して GC-MS 装置中に導入される。本方法を用いるとともに、主要なテルペン類各々について検量線を作成し定量を試みた。その結果、これまで検出できなかった微量のテルペン類についても低い検出限界と 10% 以下の変動係数で定量することができた。本 SBSE 法を用いて、強いホップ香を有するビール中のテルペン類濃度を測定したところリナロール、ゲラニオール以外のテルペン類濃度は閾値濃度に比べて極めて低いことがわかった。しかし、これらの 2 成分のみでは各品種の特徴香や、ホップ使用方法を変えたときの香調の変化を説明できず、他にホップ香を構成する成分が多く存在することが示唆された。

3. ビール中に移行するホップ由来香気成分の解析

ビールのホップ香を構成する成分を明らかにするために、通常の約5倍量のホップを用いて香り付けしたビールと、ホップを加えない無ホップビールを作り、官能評価およびGC匂い嗅ぎ分析法によって比較した。そのためにまず、ホップ品種ごとの特徴を評価するための官能評価系を確立した。この官能評価の結果、チェコ産ザーツ品種を用いたビールは柑橘、フローラルな香りを、ドイツ産ヘルスブルッカー品種を用いたビールからはグリーンでスパイシーな香りを、アメリカ産カスケード品種を用いたビールはマスカット、スグリ様の特徴を有していた。これらの特徴をもつビール、および無ホップビールから、ジクロロメタンを用いて香気成分を抽出し、GC匂い嗅ぎ分析法によって検出される香気を比較した。その結果、ホップ香を付与したビールからは検出されるが、無ホップビールからは検出されない香気が総計27種存在し、これらがビール中に残存するホップ由来の香気成分であることが分かった。そのうち19種の成分については物質を同定することができた。GC匂い嗅ぎ分析によって検出された各香気の強度から、ホップ由来のフルーティ、フローラルな香りにはエステル、テルペン、ケトン、チオール類が、グリーンな香りにはアルデヒド、アルコールが寄与していることが示唆された。また、マスカット、スグリ様の香りにはチオール基をもつ4-methyl-4-mercapto-2-pentanone (4MMP)、3-mercaptohexan-1-ol (3MH)が寄与していることが明らかになった。一方、スパイシーな香りに寄与する香気成分を検出することはできたが、同定には到らなかった。

4. 熟成ホップ使用によるエステル類の増加

ホップ使用方法の一つとして、ホップを10~40℃で一定期間熟成させた後に用いることによって、フルーティで華やかな香りを付与できることが報告されている。しかし、これに寄与する香気成分については報告されていない。この点を調べるために、ホップを40℃で30日間熟成させ、その熟成ホップを用いてビールを仕込んだ。その結果、熟成ホップを用いたビールは、官能評価において、対照(4℃保存)ホップを用いたビールと比べてフルーティな香りを有していた。前章で述べたホップ由来と確認できた27種の香気成分のうち、熟成ホップを用いることで、主にエステル類(ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl 2-methylpropanoate, ethyl 4-methylpentanoate など)が増加し、同時にグリーンな香り成分が減少していることが明らかになった。その結果、ビールのフルーティな香りを増強させることができたと考えられる。これらのエステル類は通常のビールにおいても0.1~4.0 µg/L含まれるが、熟成ホップを用いることによって2~4倍に増加し、4種の化合物は閾値を越えていた。これらのエステル類はその構造から、ホップの苦味成分である α 酸が酸化により解裂して短鎖脂肪酸を生成し、その脂肪酸が発酵中にエステル化して出来上ったものと推察した。

5. ホップ由来チオール類

前々章において4MMP、3MHなどのチオールがホップ由来の特徴香に寄与することを見出した。これらのチオール類は極めて低い閾値を持ち、ワインや果実などではその寄与が報告されている。本

研究においては、Solvent Assisted Flavor Evaporation (SAFE)、*p*-hydroxymercuribenzoate、陰イオン交換樹脂カラムを用いてチオール類を抽出し、GC-O、二次元 GC-MS を用いてチオール類の寄与と挙動を調べた。その結果、これまで検出できなかったチオール類の濃度を測定することができた。4MMP のビール中での閾値は 1.5 ng/L であり、アメリカ産カスケード品種で香り付けをしたビールには約 15 ng/L の濃度で含まれ、特徴香に寄与することがわかった。4MMP に由来するマスカット、スグリ様香気を付与するために、ホップ 17 品種中の 4MMP 濃度を測定したところ、カスケードよりも高い濃度の 4MMP を含有する 5 品種を見出した。すなわち、これらの品種は、ビールに極めて強いマスカット、スグリ様の香気を付与できることがわかった。また 4MMP は、アメリカ産、オーストラリア産、ニュージーランド産のいずれの品種にも含まれていたが、ヨーロッパ産品種には含まれていなかった。ヨーロッパ産品種に 4MMP が含まれていない理由について調べた。4MMP は 2 価の金属イオンと結合しやすい性質をもつ。ヨーロッパではベト病防止のため硫酸銅（ボルドー液）を散布しており、ヨーロッパ産ホップの銅イオン含量が極めて高かった。したがって、4MMP が銅イオンと結合し、香気を消失したと考えられる。一方、同様にマスカット、スグリ様香気をもつ 3MH はヨーロッパ産品種からも検出され、ビール中には 20~60 ng/L の濃度で含まれていた。3MH の閾値は 55 ng/L であることから、品種によっては香気に寄与していると考えられた。一部の 3MH は発酵中に、3-mercaptohexyl acetate (3MHA) に変換されていることが明らかになった。3MHA は同様にマスカット、スグリ様の香りをもち、その閾値は 3MH よりも低い (5.0 ng/L) ことを明らかにした。3MHA への変換能は酵母種によって異なると言われており、より低閾値の 3MHA への変換能が高い酵母種を選択することによって、より強い香りをもつビールを作ることができると示唆された。

6. 結 論

ホップ由来の特徴香が付与されたビール中で、その香気を組み立てる構成成分について解析した。ホップの品種、使用方法を変えたときに付与される特徴を捉え、その特徴への寄与成分を、香徴、含有濃度、閾値の観点から同定し、それらの成分のコントロール方法について検討した。これらの知見やコントロール手法は、今後、香り品質を設計することによるビールの新商品開発において、有用な手段となり得る。

References

- 1 : Acree, T. E.; Barnard, J.; Cunningham, D. G. The analysis of odor-active volatiles in gas chromatography effluents. In *Analysis of Volatiles*; Schreier, P., Ed., Walter De Gruyter: New York, **1984**; pp 251–267.
- 2 : Acree, T. E.; Barnard, J.; Cunningham, D. G. A procedure for the sensory analysis of gas chromatography effluents. *Food Chem.* **1984**, *14*, 273-286.
- 3 : Acree, T. E. Bioassays in flavor. In *Flavor Science. Sensible Principles and Techniques*; ACS Professional Reference Book; Acree, T. E., Teranishi, R., Eds.; American Chemical Society: Washington, DC, **1993**; pp 1–22.
- 4 : Acree, T. E. Gas Chromatography-olfactometry. In *Flavor Measurement*; Ho, C.-T., Manley, C. H., Eds.; Dekker: New York, **1993**; pp 77-94.
- 5 : Acree, T. E.; Barnard, J. A method for the measurement of odor. In *A Tutorial for CharmAnalysis*; DATU: New York, **1996**; pp 7–8.
- 6 : Ayers, J. E.; Fishwick, H. J.; Loud, B. G.; Swain, T. Off-flavour of Dehydrated Carrot stored in Oxygen. *Nature*, **1964**, *203*, 81–82.
- 7 : Baltussen, E.; Sandra, P.; David, F.; Cramers, C. Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: theory and practices. *J. Microcol. Sep.* **1999**, *11*, 737-747.
- 8 : Barth-Haas group “Hop varieties”; http://www.barthhaasgroup.com/cmsdk/content/bhg/hop_varieties.htm (accessed on Aug. 4, 2006).
- 9 : Belitz H.-D.; Grosch, W.; Schieberle, P. Aroma Compounds. In *Food Chemistry*. 3 rd revised edition, Springer: Berlin. **2004**. 342-408.
- 10 : Brown, D. G. W.; Clapperton, J. F.; Meilgaard, M. C.; Moll, M. Flavor thresholds of added substances. *J. Am. Soc. Brew. Chem.* **1978**, *36*, 73–80.
- 11 : Burdock, G. A. *Fenaroli's Handbook of Flavor Ingredients*, edition no. 4. CRC Press: Boca Raton, Florida, **2001**.
- 12 : Buttery, R.G.; Ling, L, C. The chemical composition of the volatile oil of hops. *Brewers' Digest*, **1966**, *41*, 71-77.
- 13 : Buttery, R. G. Flavor chemistry and odor thresholds. In *Flavor Chemistry: 30 Years of Progress*; Teranishi, R., Wick, E. L., Hornstein, I., Eds.; Kluwer Academic/Plenum Publishers: New York,

1999; pp 353-365.

- 14 : Chapman, A. C. Essential oil of hops. *J. Chem. Soc. Trans.* **1895**, 67, 54-63.
- 15 : Chapman, A. C. Essential oil of hops. *J. Chem. Soc. Trans.* **1903**, 83, 505–513.
- 16 : Chapman, A. C. The essential oil of hops. *J. Inst. Brew.* **1929**, 35, 247–255.
- 17 : Chevance, F.; Guyot-Declerck, C.; Dupont, J.; Collin, S. Investigation of the beta-damascenone level in fresh and aged commercial beers. *J. Agric. Food Chem.* **2002**, 50, 3818-3821.
- 18 : Darriet, P.; Tominaga, T.; Lavigne, V.; Boidron, N.; Dubourdieu, D. Identification of a powerful aromatic component of *Vitis vinifera* L. var. sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavour Fragrance J.* **1995**, 10, 385–392.
- 19 : David, F.; Sandra, P.; Hoffmann, A.; Harms, D.; Nietzsche, F. Elucidation of the hoppy aroma in beers by stir bar and headspace sorptive extraction followed by Thermal Desorption - CGC - MS/PFPD. *Gerstel Application Notes 4/2001*; <http://www.gerstel.com/p-gc-an-2001-04.pdf>
- 20 : De Cooman, L.; Aerts, G.; Van Opstaele, F.; Goiris, K.; Syryn, E.; De Rouck, G.; De Ridder, M.; De Keukeleire, D. New trends in advanced hopping part 2: application of varietal hop aromas. *Cerevisia Belg. J. Brew. Biotechnol.* **2004**, 29, 81–87.
- 21 : De Keukeleire, D.; David, F.; Haghebaert, K.; Sandra, P. Automated reporting on the quality of hops and hop products. *J. Inst. Brew.* **1998**, 104, 75-82.
- 22 : Deinzer, M.; Yang, X. Hop aroma: character impact compounds found in beer, methods of formation of individual compounds. In *EBC Monograph XXII. Symposium on Hops, Zoeterwoude, The Netherlands*; Getränke-Fachverlag Hans Carl: Nürnberg, Germany, **1994**; pp 181-197.
- 23 : Delahunty, C. M.; Eyres, G.; Dufour, J.-P. Gas chromatography-olfactometry. *J. Sep. Sci.* **2006**, 29, 2107-2125.
- 24 : Demyttenaere, J. C. R.; Sanchez Martinez, J. I.; Verhe, R.; Sandra, P.; De Kimpe, N. Analysis of volatiles of malt whisky by solid-phase microextraction and stir bar sorptive extraction. *J. Chromatogr.* **2003**, 985, 221-232.
- 25 : E.B.C.–Analytica Microbiologica *J. Inst. Brew.* **1977**, 83, 109–118.
- 26 : European Brewery Convention, *Hops and Hop Products, Manual of Good Practice*; EBC Technology and Engineering Forum; Getränke-Fachverlag Hans Carl: Nürnberg, Germany, **1997**.
- 27 : Engel, H. K.; Tressl, R. Identification of new sulfur-containing volatiles in yellow passion fruit (*Passiflora edulis f. Flavicarpa*). *J. Agric. Food Chem.* **1991**, 39, 2249–2252.
- 28 : Engel, E.; Nickerson, G.. Use of the hop aroma component profile to calculate hop rates for standardizing aroma units and bitterness units in brewing. *J. Am. Soc. Brew. Chem.* **1992**, 50, 82-22.

- 29 : Engel, W.; Bahr, W.; Schieberle, P. Solvent assisted flavour evaporation - a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *Eur. Food Res. Technol.* **1999**, 209, 237–241.
- 30 : European Brewery Convention. Lead conductance value of hops, powders and pellets, *Analytica-EBC*, 4th ed. EBC: Zurich, Switzerland, **1987**. Hops-7.4
- 31 : Eyres, G.; Dufour, J. P.; Hallifax, G.; Sotheeswaran, S.; Marriot, P. Identification of character-impact odorants in coriander and wild coriander leaves using gas chromatography-olfactometry (GCO) and comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GC × GC-TOFMS). *J. Sep. Sci.* **2005**, 28, 1061-1074.
- 32 : Eyres, G.; Marriott, P. J.; Dufour, J. P. Comparison of Odor-Active Compounds in the Spicy Fraction of Hop (*Humulus lupulus* L.) Essential Oil from Four Different Varieties. *J. Agric. Food Chem.* **2007**, 55, 6252 -6261.
- 33 : Fritsch, H. T.; Schieberle, P. Identification based on quantitative measurements and aroma recombination of the character impact odorants in a Bavarian Pilsner-type beer. *J. Agric. Food Chem.* **2005**, 53, 7544–7551.
- 34 : Fukuoka, Y.; Kowaka, M. Identification of compounds imparting hoppy flavour to beer. *Rep. Res. Lab. Kirin Brew. Co.* **1983**, 26, 31-36.
- 35 : Gardner, D. Hop flavour and aroma products. In *EBC Monograph XXII. Symposium on Hops, Zoeterwoude, The Netherlands*; Getränke-Fachverlag Hans Carl: Nürnberg, Germany, **1994**; pp 114-126.
- 36 : Goiris, K.; De Ridder, M.; De Rouck, G.; Boeykens, A.; Van Opstaele, F.; Aerts, G.; De Cooman, L.; De Keukeleire, D. The oxygenated sesquiterpenoid fraction of hops in relation to the spicy hop character of beer. *J. Inst. Brew.* **2002**, 108, 86–93.
- 37 : Grosh, W. Evaluation of the key odorants of food by dilution experiments, aroma models and omission. *Chem. Senses*, **2001**, 26, 533-545.
- 38 : Guadagni, D.G., Buttery, R.G., and Harris, J. Odour intensities of hop oil components. *J. Sci. Food Agric.* **1966**, 17, 142-144.
- 39 : Hanin, L. *Cours de Matière Médicale, par D; Hanin*: Paris, **1819**; T. 1. pp 332.
- 40 : Hatanaka, A. Biosynthesis of so-called “green odor” emitted by green leaves. In *Comprehensive Natural Products Chemistry*; Elsevier Science: Oxford, U. K., **1999**; Vol. 1, pp 83–116.
- 41 : Head Retention.; http://www.brewwiki.com/index.php/Head_Retention (accessed on Jan. 22, 2008).

- 42 : Hildebrand, R. P.; Kavanagh, T. E.; Clarke, B. J. Hop lipids and beer quality. *Brew. Dig.* **1975**, 50, 58–60.
- 43 : Hopsteiner “Hop Products: Iso-Extract.”; <http://www.hopsteiner.com/isopgl.htm> (accessed on Jan. 22, 2008).
- 44 : Hopsteiner “Hop varieties”; <http://www.hopsteiner.com/varieties.html> (accessed on Jan. 22, 2008).
- 45 : Howard, G. A. New approach to the analysis of hop oil. *J Inst. Brew.* **1956**, 62, 158-159.
- 46 : Howard, G. A. Evaluation of hops VII. Composition of the essential oil of hops. *J Inst. Brew.* **1957**, 63, 491-506.
- 47 : Irwin, A.J. Novel approaches for isolating hop flavour constituents of beer. *Proc. Eur. Brew. Conv.* **1985**, 587-594.
- 48 : Irwin, A. J. Varietal dependence of hop flavour volatiles in lager. *J. Inst. Brew.* **1989**, 95, 185-194.
- 49 : Isoe, S.; Katsumura, S.; Sakan, T. The synthesis of damascenone and beta-damascone and the possible mechanism of their formation from carotenoids. *Helv. Chim. Acta.* **1973**, 56, 1514-1516.
- 50 : Japan Aroma Society *Kaori no Hyakka (Encyclopedia of Aromas)*, Asakura Shoten, Japan, **1989**.
- 51 : Johansen, N.; Plam, M. and Legier, M. Detection of sulfur compounds in hop oils with a novel sulfur-selective detector for gas chromatography. *Brew. Dig.* **1990**, 65, 36-37.
- 52 : Kač, M.; Kovačević, M. Presentation and determination of hop (*Humulus lupulus* L.) cultivars by a min-max model on composition of hop essential oil. *Monatsschr. Brau.* **2000**, 53, 180–184.
- 53 : Kaltner, D.; Mitter, W. The influence of hops upon the aroma and taste of beer. *Scand. Brew. Rev.* **2003**, 60, 8–16.
- 54 : King, A. J.; Dickinson, J. R. Biotransformation of hop aroma terpenoids by ale and lager yeasts. *FEMS Yeast Res.* **2003**, 3, 53-62.
- 55 : Kobayashi, H.; Tominaga, T. New style of Kosshu wine –Kiiko-ka-. *J. ASEV Jpn.* **2005**, 16, 150–151.
- 56 : Kotseridis, Y.; Baumes, R. L.; Skouroumounis, G. K. Quantitative determination of free and hydrolytically liberated beta-damascenone in red grapes and wines using a stable isotope dilution assay. *J. Chromatogr. A.* **1999**, 849, 245-254.
- 57 : Kowaka, K.; Fukuoka, Y.; Kawasaki, H.; Asano, K. The true value of aroma hops in brewing. *Proc. Eur. Brew. Conv.* **1983**, 71-78.
- 58 : Kowaka, M.; Fukuoka, Y. Identification of compounds imparting hoppy flavor to beer. *Brew. Dig.* **1985**, 8, 46-48.

- 59 : Kunze, W. *Technology brewing and malting, English translation of the 7th.*; VLB Berlin Publication: Germany, **1997**.
- 60 : Lam, K. C.; Foster, R. T.; Deinzer, M. L. Aging of hops and their contribution to beer flavor. *J. Agric. Food Chem.* **1986**, *34*, 763-779.
- 61 : Lermusieau, G.; Bulens, M.; Collin, S. Use of GC-olfactometry to identify the hop aromatic compounds in beer. *J. Agric. Food Chem.* **2001**, *49*, 3867-3874.
- 62 : Maier, J. Zusammensetzung des Hopfenöls in Beziehung zu Sorte und Qualität des Hopfens und seinen Umwandlungsprodukten. *Monatsschr. Brau.* **1966**, *19*, 425-434.
- 63 : Masuda, S.; Kikuchi, K.; Harayama, K.; Sakai, K.; Ikeda, M. Determination of lightstruck character in beer by gas chromatography–mass spectroscopy. *J. Am. Soc. Brew. Chem.* **2000**, *58*, 152–154.
- 64 : Maylan, M. W.; Howard, P. H. Atom/Fragment contribution method for estimating octanol-water partition coefficients. *J. Pharm. Sci.* **1995**, *84*, 83-92.
- 65 : Meilgaard, M. C. Flavor chemistry of beer: part ii: flavour and threshold of 239 aroma volatiles. *Tech. Q. Master Brew. Assoc. Am.* **1975**, *12*, 151–168.
- 66 : Meilgaard, M.C.; Civille, G.V.; Carr, B.T. *Sensory Evaluation Technique*, 4th ed. CRC Press: Boca Raton, Florida, **1999**.
- 67 : Moir, M. Hops-a millennium review. *J. Am. Soc. Brew. Chem.* **2000**, *58*, 131-146.
- 68 : Murakami, A.; Goldstein, H.; Chicoye, E. Development of a high-resolution beer headspace analysis method. *J. Am. Soc. Brew. Chem.* **1986**, *44*, 33-37.
- 69 : Narziss, L.; Miedaner, H.; Gresser, A. Comparison of the hop oil spectrum of different varieties of hops. *Brauwelt Int. (Eng. Issue)* **1986**, *1*, 17-22.
- 70 : New Zealand Hops Limited. “Hop varieties”; <http://nzhops.co.nz/varieties/index.html> (accessed on Jan. 22, 2008).
- 71 : Nickerson, G.; Van Engel, L. Hop aroma component profile and the aroma unit. *J. Am. Soc. Brew. Chem.* **1992**, *50*, 77-81.
- 72 : Nijssen, L.; Vissher, C.; Maarse, H.; Willemsens, L.; Boelens, M. *Volatile Compounds in Food: Qualitative and Quantitative Data*, 7th ed.; TNO Nutrition and Food Research Institute: Zeist, The Netherlands, **1996**.
- 73 : Ochiai, N.; Sasamoto, K.; Daishima, S.; Heiden, A. C.; Hoffmann, A. Determination of stale-flavor carbonyl compounds in beer by stir bar sorptive extraction with *in-situ* derivatization and thermal desorption-gas chromatography-mass spectrometry. *J. Chromatogr.* **2003**, *986*, 101-110.

- 74 : Ono, M.; Kakudo, Y.; Yamamoto, Y.; Nagami, K.; Kumada, J. Simultaneous Analysis of Hop Bittering Components by High-Performance Liquid Chromatography and Its Application to the Practical Brewing. *J. Am. Soc. Brew. Chem.* **1985**, *43*, 136–144.
- 75 : Peppard, T. L.; Ramus, S. A.; Witt, C. A.; Siebert, K. J. Correlation of sensory and instrumental data in elucidating the effect of varietal differences on hop flavor in beer. *J. Am. Soc. Brew. Chem.* **1989**, *47*, 18-26.
- 76 : Perpète, P.; Mélotte, L.; Dupire, S.; Collin, S. Varietal discrimination of hop pellets by essential oil analysis. I. Comparison of fresh samples. *J. Am. Soc. Brew. Chem.* **1998**, *56*, 104–108.
- 77 : Pickett, J. A.; Coates, J.; Sharpe, F. R. Improvement of hop aroma in beer. *Proc. Eur. Brew. Conv.* **1975**, 123-140.
- 78 : Rabak, F. Aroma of Hops: A study of the volatile oil with relation to the geographical sources of the hops. *J. Agric. Research* **1914**, *2*, 115-159.
- 79 : Roberts, M. T.; Dufour, J.-P.; Lewis, A. C. Application of comprehensive multidimensional gas chromatography combined with time-of-flight-mass spectrometry (GC×GC-TOFMS) for high resolution analysis of hop essential oil. *J. Sep. Sci.* **2004**, *27*, 473-478.
- 80 : Sanchez, N. B.; Lederer, C. L.; Nickerson, G. B.; Libbey, L. M.; McDaniel, M. R. Sensory and analytical evaluation of hop oil oxygenated fractions. In *Food Science and Human Nutrition*; Charalambous, G., Ed.; Elsevier Science: Amsterdam, **1992**; pp 371-402.
- 81 : Sanderson, G. W.; Gonzales, J. G. Biochemistry of tea fermentation: the role of carotenes in black tea aroma formation. *J. Food Sci.* **1971**, *36*, 231–236.
- 82 : Sanderson, G. W.; Graham, H. N. Formation of black tea aroma. *J. Agric. Food Chem.* **1973**, *21*, 576–585.
- 83 : Scheffé, H. An analysis of variance for paired comparisons, *J. Amer. Stat. Assoc.* **1952**, *147*, 381-400.
- 84 : Seaton, J.; Moir, M.; Suggett, A. The refinement of hop flavour by yeast action. *Curr. Dev. Malt. Brew. Distill. (Proc. Aviemore Conf., 1st)*, **1983**, 111-128.
- 85 : Schieberle, P. Primary odorants of pale lager beer. Differences to other beers and changes during storage. *Z. Lebensm. Unters. Forsch.* **1987**, *185*, 111–113.
- 86 : Schieberle, P. Recent developments in methods for analysis of flavor compounds and their precursors. In *Characterization of Food: Emerging Methods*. Gaonkar, A. G., Eds.; Elsevier Science: Amsterdam, **1995**; pp 403–431.
- 87 : Sharpe, F. R.; Chappell, C. G. An introduction to mass spectrometry and its application in the analysis of beer, wine, whisky and food. *J. Inst. Brew.* **1990**, *96*, 381-393.

- 88 : Silbereisen, K.; Kruger, K.; Wagner, B.; Forch, M. Einfluss einiger hopfenaolkomponenten auf geschmack und aroma des bieres. *Monatsschr. Brau.* **1968**, *206*, 206-211.
- 89 Steinhaus, M.; Schieberle, P. Comparison of the most odor-active compounds in fresh and dried hop cones (*Humulus lupulus* L. variety spalter select) based on GC-olfactometry and odor dilution techniques. *J. Agric. Food Chem.* **2000**, *48*, 1776–1783.
- 90 : Steinhaus, M.; Fritsch, H.T.; Schieberle, P. Quantitation of (*R*)- and (*S*)-linalool in beer using solid phase microextraction (SPME) in combination with a stable isotope dilution assay (SIDA). *J. Agric. Food Chem.* **2003**, *51*, 7100–7105.
- 91 : Steinhaus, M.; Schieberle, P. Untersuchungen zum Einfluss der Hopfensorte auf das Aroma hopfenbetonter Biere unter besonderer Berücksichtigung glycosidisch gebundener Hopfenaromastoffe.; <http://dfal.de/aif-fv13622n.html> (accessed Aug. 4, 2006).
- 92 : Steinhaus, M.; Schieberle, P. Transfer of the potent hop odorants linalool, geraniol and 4-methyl-4-sulfanyl-2-pentanone from hops into beer. *Proc. Eur. Brew. Conv.* **2007**, 112.
- 93 : Steinhaus, M.; Wilhelm, W.; Schieberle, P. Comparison of the most odour-active volatiles in different hop varieties by application of a comparative aroma extract dilution analysis. *Eur. Food Res. Technol.* **2007**, *226*, 45-55.
- 94 : Sub-Committee Report Sensory Analysis. *J. Am. Soc. Brew. Chem.*, **1980**, *38*, 99–107.
- 95 : Swiegers, J.H.; Francis, I.L.; Herderich, M.J.; Pretorius, I.S. Meeting consumer expectations through management in vineyard and winery - the choice of yeast for fermentation offers great potential to adjust the aroma of Sauvignon Blanc wine. *Aust. N.Z. Wine Ind. J.*, **2006**, *21*, 34–42.
- 96 : Swiegers J.H.; Capone D.L.; Pardon K.H.; Elsey G.M.; Sefton M.A.; Francis I.L.; Pretorius I.S. Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. *Yeast*. **2007**, *24*, 561-574.
- 97 : Tominaga, T.; Masneuf, I.; Dubourdieu, D. A S-cysteine conjugate, precursor of aroma of white Sauvignon. *J. Int. Sci. Vigne Vin.* **1995**, *29*, 227-232.
- 98 : Tominaga, T., Furrer, A., Henry, R., and Dubourdieu, D. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. *Flavour Fragrance J.* **1998**, *13*, 159–162.
- 99 : Tominaga, T.; Baltenweck-Guyot, R.; Peyrot de Gachons, C.; Dubourdieu, D. Contribution of volatile thiols to the aromas of white wines made from several *Vitis vinifera* grape varieties. *Am. J. Enol. Vitic.* **2000**, *51*, 178–181.
- 100 : Tominaga, T.; Masneuf, I.; Dubourdieu, D. Powerful aromatic volatile thiols in wines made from several *Vitis vinifera* grape varieties and their releasing mechanism. *ACS Symposium Series*

- 871, Shahidi, F., Weerasinghe, D. K., Eds.; American Chemical Society: Washington, DC, **2003**; pp 314–337.
- 101 : Tominaga, T.; Dubourdieu, D. A novel method for quantification of 2-Methyl-3-furanthiol and 2-Furanmethanethiol in wines made from *Vitis vinifera* grape Varieties. *J. Agric. Food Chem.* **2006**, *54*, 29–33.
- 102 : Tominaga, T., Niclass, Y., Frerot, E., and Dubourdieu, D. Stereoisomeric distribution of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate in dry and sweet white wines made from *Vitis vinifera* (Var. Sauvignon Blanc and Semillon). *J. Agric. Food Chem.* **2006**, *54*, 7251-7255.
- 103 : Tressl, R.; Friese, L.; Fendesack, F.; Koppler, H. Studies of the volatile composition of hops during storage. *J. Agric. Food Chem.* **1978**, *26*, 1426-1430.
- 104 : Tressl, R.; Friese, L.; Fendesack, F.; Kruger, E. Free fatty acids in hops - A criterion for differentiation of variety and assessment of degree of aging. *Monatsschr. Brau.* **1978**, *31*, 83-86.
- 105 : Ullrich, F.; Grosch, W. Identification of most intense volatile flavour compounds formed during autoxidation of linoleic acid. *Z. Lebensm. Unters. Forsch.* **1987**, *184*, 277–282.
- 106 : Vermeulen, C.; Guyot-Declerck, C.; Collin, S. Combinatorial synthesis and sensorial properties of mercapto primary alcohols and analogues. *J. Agric. Food Chem.*, **2003**, *12*, 3623–3628.
- 107 : Vermeulen, C.; Lecocq, S.; Collin, S. Polyfunctional thiols and drinkability of beer. *Proc. Eur. Brew. Conv.* **2003**, 91/1-91/11.
- 108 : Vermeulen, C.; Lejeune, I.; Tran, T.; Collin, S. Occurrence of polyfunctional thiols in fresh lager beer. *J. Agric. Food Chem.* **2006**, *54*, 5061–5068.
- 109 : Walker, M. D. The influence of metal ions on concentrations of flavour-active sulphur compounds measured in beer using dynamic headspace sampling. *J. Sci. Food Agric.* **1995**, *67*, 25-28.
- 110 : Yakima Chief, Inc. “High quality hops from Yakima valley”; <http://www.yakimachief.com/hopvarieties/hopvar.html> (accessed on Jan. 22, 2008).

List of Publications

The contents of current study were included in the following publications;

Chapter 1.

- Kishimoto, T.; Wanikawa, A.; Kagami, N.; Kawatsura, K. Analysis of hop-derived terpenoids in beer and evaluation of their behavior using the stir bar-sorptive extraction method with GC-MS. *J. Agric. Food Chem.* **2005**, *53*, 4701-4707.

Chapter 2.

- Kishimoto, T.; Wanikawa, A.; Kono, K.; Shibata, K. Comparison of the odor-active compounds in unhopped beer and beers hopped with different hop varieties. *J. Agric. Food Chem.* **2006**, *54*, 8855–8861.

Chapter 3.

- Kishimoto, T.; Kobayashi, M.; Yako, N.; Iida, A.; Wanikawa, A. Comparison of 4-mercapto-4-methylpentan-2-one content in hop cultivars from different growing regions. *J. Agric. Food Chem.* **2008**, *56*, 1051–1057.

Chapter 4.

- Kishimoto, T.; Morimoto, M.; Kobayashi, M.; Yako, N.; Iida, A.; Wanikawa, A. Behaviors of 3-mercaptohexan-1-ol, 3-mercaptohexyl acetate during brewing processes. *J. Am. Soc. Brew. Chem.* **2008**, *66*, 192-196.

Chapter 5.

- Kishimoto, T.; Wanikawa, A.; Kono, K.; Aoki, K. Odorants comprising hop aroma of beer: hop-derived odorants increased in the beer hopped with aged hops. In *Proceedings of the 31st European Brewery Convention Congress*; Venice, Italy, **2007**; pp 226-235.

Other publications

- Kishimoto, T. Beer flavor analysis with a Twist. In *GERSTEL Solutions Worldwide*, Gerstel: Mulheim a/d Ruhr, Germany, May **2007**; pp 3-7.
- Kishimoto, T.; Ozaki, K.; Wanikawa, A. Method of sensory analysis for beer. *J. Japan Association on Odor Environment*, **2007**, 38, 361-367.
- Kishimoto, T. Investigations of hop-derived odor-active components in beer. In *Proceedings of "1st International Brewers Symposium, - Hop Flavor and Aroma"*, Master Brewers Association of the Americas: Minnesota, **2007**.

Conference Presentations

- Kishimoto, T.; Kagami, N.; Kawatsura, K. Analysis of hop terpenes in beer and wort using the SBSE method with GC-MS. *World Brewing Congress*: San Diego, USA, **2004**.
- Kishimoto, T.; Wanikawa, A.; Kono, K.; Aoki, K. Odorants comprising hop aroma of beer : hop-derived odorants increased in the beer hopped with aged hops. *31st Congress of the European Brewery Convention*: Venice, Italy, **2007**.
- Kishimoto, T. Investigations of hop-derived odor-active components in beer. *1st International Brewing Symposium*: Corvallis, USA, **2007**.

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